COMPARATIVE ANALYSIS OF GUT MICROBES IN ANEMIA MODEL

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

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IN

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I hereby declare that the work which is being presented in this dissertation entitled "**Comparative Analysis of Gut Microbes in Anemia Model**" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from 2nd January, 2025 to 31st May, 2025 under the supervision of **Dr. Mausumi Sikdar, Department of Life Sciences, Presidency University, Kolkata**.

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ABSTRACT

The gut microbiota is essential for maintaining host health because it aids in digestion, nutrient absorption, immunological control, and defense against pathogens. Changes in the makeup of the gut microbiota have been related to a variety of illnesses, such as hematological and metabolic problems. Low red blood cell count or hemoglobin concentration, which is the defining feature of anemia, can affect the gut flora and, as a result, the makeup of the intestinal bacteria. The goal of this study is to compare the gut microbiome of three anemic mice with that of one healthy, non-anemic control mouse in order to find potential variations in bacterial diversity and traits.

Bacterial extracts were taken from the gut contents of each mouse under sterile conditions in order to carry out the analysis. These extracts were then incubated for 24 hours at 37°C on nutrient agar (NA) plates using spread plating. Based on their physical features, such as size, form, color, and texture, the bacterial colonies that grew were observed and classified. These findings gave early hints about the variety of microbial communities found in the gut.

The metabolic characteristics of the isolated bacteria were further determined by a variety of biochemical assays. In order to isolate Gram-negative enteric bacteria and differentiate lactose fermenters, the isolates were also grown on MacConkey agar. The Kirby-Bauer disc diffusion method was also used to assess the isolates' antibiotic susceptibility profiles. By comparing the microbial profiles of anemic and healthy mice, this study aims to identify potential alterations in the composition of the gut bacteria associated with anemia, furthering our knowledge of the relationship between gut health and disease.

KEYWORDS

Anemia, IMViC, Kirby Bauer, MacConkey, Gram staining.

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ABBREVIATIONS

Abbreviation	Full form
NA	Nutrient Agar
NB	Nutrient broth
MR	Methyl Red
VP	Vogesprokauer
TSI	Triple Sugar Iron
R	Resistant
S	Sensitive
ROS	Reactive Oxygen Species
EDTA	Ethylenediaminetetraacetic Acid
MSCRAMM	Microbial Surface Components Recognizing Adhesive Matrix Molecule
CV	Crystal Violet
CVI	Crystal Violet Iodine Complex
DMAB	Para-dimethylaminobenzaldehyde
LBL	Left back leg
RBL	Right back leg
B	Body
T	Tail
RFL	Right front leg
IWB	White, big, Ileum
IWM	White, medium, Ileum
IWS	White, small, Ileum
IY	Yellow, Ileum
CWB	White, big, Colon
CWM	White, medium, Colon
CWS	White, small, Colon
CY	Yellow. Colon
IWB	White, big, Ileum

IO	Orange, Ileum
IWS	White, small, Ileum
IY	Yellow, Ileum
CWB	White, big, Colon
CO	Orange, Colon, Colon
CWS	White, small, Colon
CY	Yellow, Colon
IWB	White, big, Ileum
IWS	White, small, Ileum
CWB	White, big, Colon
CWS	White, small, Colon
FWB	White, big, Feces
FWS	White, small, Feces

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INTRODUCTION

The gastrointestinal tract of mammals, including mice, is home to a diverse and complex microbial community that is essential to many biological functions, such as digestion, immune system regulation, and defense against harmful pathogens. The makeup and features of the gut flora of four lab mice (three anemic and one control) were examined in an experiment conducted in the current study. Three mice were taken, LBL+RBL, B+T+LBL, B+T+RFL were first made anemic by inducing it with Phenylhydrazine. A non-diseased control mouse was taken, LBL+RBL. The main goal was to isolate and identify the cultivable bacteria in the gut by extracting their intestinal contents, culturing the microorganisms on different media, and observing the resulting colonies. The use of culture-based methods, despite their limitations in identifying uncultivable species, is still necessary for identifying viable and functional bacterial groups.

The gut bacterial contents were aseptically extracted after the mice were euthanized in a humane and sterile manner. After that, 100 microlitre of bacterial extracts were spread-plated on nutrient agar (NA) plates, which are a versatile medium that promotes the growth of a wide variety of non-fastidious species. Following a 24-hour incubation period at 37°C, which is ideal for the proliferation of many mesophilic gut bacteria, several different colonies began to grow on the agar surface. Based on characteristics including color, size, form, height, and margin, these colonies were morphologically analyzed, providing basic hints as to their identity and variety.

A battery of biochemical assays was performed in order to further identify the isolates. These tests sought to ascertain particular metabolic and enzymatic processes, which would allow for the identification of bacterial genera and species according to their physiological characteristics. Among the typical biochemical experiments were Indole test, Methyl-red test, Voges Proskauer test, Citrate utilization test, Urease test, Catalase test, Coagulase test, Triple sugar Iron (TSI), along with Gram staining was performed. Additionally, the isolates were grown on MacConkey agar, a selective and differential media intended to isolate Gram-negative rods and differentiate between lactose fermenters and non-fermenters according to the colony's color and growth patterns.

Additionally, the antibiotic susceptibility profiles of the isolated strains were evaluated using the Kirby-Bauer disk diffusion technique. Using this method, the test organisms are incubated on agar plates that have been infected with disks impregnated with antibiotics. The susceptibility or resistance of the bacteria to particular antibiotics is indicated by the presence and size of the inhibitory zones surrounding the disks. Considering the growing antibiotic resistance, this element of the research is especially significant since gut microorganisms may act as reservoirs for resistant genes.

This study offers a fundamental knowledge of the microbial population in the mouse gut through its comprehensive methodology, which includes microbiological culturing, colony characterization, biochemical identification, selective media growth, and antibiotic susceptibility testing. The results may shed light on future research on gut health, probiotic creation, and infectious disease treatment, as well as add to the body of evidence that backs studies in microbiology, immunology, and pharmacology.

REVIEW OF LITERATURE

Spread plating of 100 microlitre of bacterial extract from the gut of the three anemic mice and one control mouse was done. Several biochemical tests like Indole test, Methyl-red test, Voges Proskauer test, Citrate utilization test, Urease test, Catalase test, Coagulase test, Triple sugar Iron (TSI) test along with other tests like Kirby Bauer test, growing them on MacConkey agar and Gram staining were performed to identify the bacteria present in the gut of these four mice. All this was keeping in mind the appearance of the bacterial colonies observed with naked eye, on the Nutrient agar plates or MacConkey agar plates that they were grown.

The indole test determines if an organism can make indole, also known as benzyl pyrole, a tryptophan byproduct. Tryptophanase-producing bacteria hydrolyze tryptophan and generate ammonia, pyruvic acid, and indole. Bacteria that express the tryptophanase enzyme are able to hydrolyze and deaminate the amino acid tryptophan.

When indole interacts with the aldehyde group of para-dimethylaminobenzaldehyde (DMAB) in an acidic environment, a quinoidal red complex (rosindole) or cherry red ring is formed. This is the active ingredient in Kovác's indole detection reagent. The reagent's amyl alcohol aids in indole's trapping. *Enterobacter* show indole negative, while *Escherichia coli* show indole positive (cherry red ring).

After a few seconds of applying Kovác's reagent, if a cherry red ring appears at the reagent-broth interface, it means that tryptophanase is being produced and indole is present, else if there is no cherry red ring, tryptophanase is not formed, which means indole negative result. To perform this test, a tryptophan-rich media is needed.

Methyl red is an indicator. It is used in the methyl-red test and helps to determine if a bacteria has a mixed acid fermentation pathway. Methyl red is introduced to the MRVP broth inoculation tube to serve as a pH indicator. *Escherichia coli* creates stable acidic end products using a mixed acid fermentation pathway. The medium's buffers will be overpowered by the acids, creating an acidic environment. The pH now hovers around 4.4. Methyl red doesn't change color when exposed to acidic conditions. As is the case with Pseudomonas sp. and

Bacillus cereus, it produces orange or yellow coloring when there is less or no acid production, which is considered to be a bad outcome.

The Voges-Proskauer (VP) test is a biochemical assay utilized to detect enteric bacteria such as *Escherichia, Enterobacter, and Klebsiella*. This test identifies the presence of acetoin, a byproduct formed during glucose metabolism through a particular enzymatic pathway. For the test, a bacterial culture is treated with alpha-naphthol and potassium hydroxide, resulting in a red color due to the reaction with acetoin. For Klebsiella, Enterobacter, Hafnia, and Serratia, acetoin is usually the main final product of glucose metabolism. Nonetheless, Klebsiella can display both MR+ and VP+ traits based on the surroundings. It is essential to understand that 2,3-butanediol is difficult to detect in this assay because of its neutral characteristics, hence the emphasis is on identifying acetoin or acetyl methyl carbinol, which transforms into diacetyl with the introduction of alpha-naphthol and KOH (Barritt's reagent).

The citrate test is performed to detect bacteria that use citrate as their only carbon source. Simmone's citrate medium, utilized for this test, consists of citrate as the only carbon source. Bacteria that thrive on this medium generate an enzyme, citrate-permease, which can transform citrate into pyruvate. Pyruvate may subsequently enter the metabolic cycle of the organism to generate energy. Growth indicates the use of citrate, a key intermediate in the Krebs cycle. As bacteria uses the citrate, the ammonium salts break into ammonia, leading to a rise in alkaline environment. The change in pH changes the bromothymol blue indicator in the medium from green to blue. The only nitrogen source in the medium is Ammonium dihydrogen phosphate. Dipotassium phosphate functions as a buffer. Sodium chloride preserves the osmotic equilibrium of the solution. Sodium citrate is the only carbon source in this medium. Magnesium derived from magnesium sulfate acts as a cofactor for numerous metabolic reactions. The solidifying agent is bacteriological agar. If citrate is utilized, the microbe will gather alkaline/basic metabolites.

Urease enzyme breaks urea into ammonia and carbon dioxide. The production of urease by a microbe is utilized to identify them through biochemical testing. The pH rises as bacteria breaks the urea, which can be identified by the pH indicator Phenol red, which shows colour change to pink when in alkaline medium. Urease production serves as a means for Helicobacter pylori to develop opportunistic pathogenesis, utilizing a micro-alkaline environment to shield itself from the stomach's highly acidic conditions, thereby facilitating the formation of gastric ulcers.

Bacteria that produce urease include *Staphylococcus epidermidis*. Urease production is seen by a vibrant pink (fuchsia) color on the slant.

The Triple Sugar Iron (TSI) test uses TSI agar as a tubed differential medium for identifying carbohydrate fermentation and H2S synthesis. In addition, gas produced by carbohydrate metabolism can be identified. TSI distinguishes bacteria based on their ability to ferment lactose, glucose, and sucrose, as well as on the creation of hydrogen sulfide.

Glucose, sucrose, and lactose are the three sugars that make up TSI. The TSI differential medium consists of 1% lactose, 1% sucrose, 0.1% glucose, ferrous sulfate, and phenol red as a pH indicator. The capacity of test microbes to use lactose, glucose, and sucrose sugar is shown by the change in the medium's color. In an acidic environment, phenol red turns yellow, while in an alkaline environment, it turns red. To identify Gram-negative intestinal bacteria, Russell employed an agar medium containing two sugars. The third sugar sucrose is later added to this dual sugar medium to change its composition. Additionally, triple sugar medium is used to isolate enteric bacilli using ferrous sulphate which reduce sulphur, and generate carbohydrate. The TSI test is primarily used to identify coliforms because they ferment sucrose faster than lactose. The butt of the test tube is yellow, while the slant is pink, if just glucose is fermented, which results in the production of enough acid. If the test bacteria process acidic by fermenting lactose, sucrose, or both, yellow butt and slant will be observed.

Catalase test is a quick assay. Catalase is found in the bacteria. The 3% hydrogen peroxide is applied externally. The production of catalase by the microorganism, makes hydrogen peroxide break into oxygen and water. Effervescence may be seen.

Catalase should be present in aerobic bacteria but not in anaerobic bacteria. Catalase allows one to differentiate between Gram-positive cocci - *Staphylococcus aureus* is catalase positive, whereas *Streptococcus* spp. is catalase negative. The purpose of the catalase enzyme is to counteract the antibacterial effects of hydrogen peroxide.

For coagulase test, the slide test will reveal clusters of cells. A positive reaction is indicated by clotting. Similarly, there is no correlation between the rate of coagulation and the virulence of the pathogen. A clean slide is used to conduct the slide test by creating a suspension of bacterial cells in a drop of human or rabbit plasma that has been treated with EDTA. The plasma will

cause the bacterial cells to cluster if they contain bound coagulase. Due to the clumping factor being an adhesin, a member of the MSCRAMM family of adhesion molecules, the cells will bind to fibrinogen in the plasma, resulting in clumping. This will cause the bacterial cells to clump together noticeably on the microscope slide.

The Gram stain is essential for determining the phenotypic characteristics of bacteria. According to cell wall structure, the staining technique distinguishes species in the domain Bacteria. Due to their dense peptidoglycan layer, gram-positive cells appear purple after being stained. Since Gram-negative cells have a thin peptidoglycan layer, they stain reddish/orangish. Crystal violet dissociates into CV+ and Cl- ions in aqueous solutions, which can pass through the cell walls and membranes of both type of cells. When iodine (I- or I3 -) is introduced, it interacts with CV+ to create big CVI complexes inside the cytoplasm and outside layers of the cell. The CV+ stains the cells purple by interacting with negatively charged elements of bacterial cells. The decolorizing agent, which is either ethanol or a combination of ethanol and acetone, interacts with the lipids in the membranes of both Gram-positive and Gram-negative bacteria. The gram-negative cell's outer membrane comes off, leaving the peptidoglycan layer exposed. Ethanol makes Gram-negative cell walls permeable, so the big CV-I complexes goes out of the cell. The addition of ethanol dehydrates the gram-positive cell's heavily cross-linked and multilayered peptidoglycan. The multi-layered aspect of the peptidoglycan, along with the dehydration caused by the ethanol treatment, traps the large CV-I complexes inside the cell. The gram-positive cell retains its purple color following decolorization, but the gram-negative cell loses it and is only seen when the positively charged dye safranin is introduced as a counterstain.

The Kirby-Bauer technique or disc diffusion is an antibiotic susceptibility test that helps decide which antibiotic to use while treating an infection. In this method, filter paper circles with a 6mm diameter are saturated with known amounts of various antimicrobial chemicals. The test organism is introduced onto Nutrient Agar (NA) plates, and these disks are positioned on top of the plates. The antimicrobial molecule will diffuse at the same time that the test organism grows. The capacity of that antimicrobial molecule to prevent the growth of the test organism is determined by whether or not the organism grows around the discs.

The following variables influence how quickly the antibacterial substance diffuses:

● The molecular weight of the antimicrobial substance. The size of the molecules is inversely proportional to their diffusion rate on the agar.
● The drug's solubility and diffusion characteristics in agar media.

Gram-negative rods belonging to the Enterobacteriaceae family, can be grown on MacConkey and hence can be distinguished where MacConkey agar acts as a differential and selective medium. It contains bile salts and crystal violet, which stop the growth of gram-positive bacteria and promoting the growth of gram-negative bacilli. Lactose fermenters that produce reddish pinkish colonies and non-fermenters that produce colorless or transparent colonies can be distinguished using MacConkey agar.

From the gut samples of four mice, a total of 23 different bacterial species were identified. Among the mice and the sampled gut regions (ileum, colon, and feces where applicable), the distribution of these strains varied greatly. From the ileum region of the first mouse (LBL+RBL), only one strain was discovered, and none were discovered in the colon. With four strains identified from the ileum and another four from the colon, the second mouse (B+T+LBL) had greater microbial diversity. The third mouse (B+T+RFL) likewise displayed four strains from each of the ileum and colon, demonstrating a well-balanced and diverse microbial community in both locations. The fourth mouse (control, LBL+RBL) exhibited moderate diversity, with two strains isolated from the ileum, two from the colon, and two additional strains from its feces. The significant variation in gut bacterial makeup between the individual mice revealed by this comparative distribution may be related to physiological variables, such as disease state, notably anemia in three of the four mice. The first mouse's extraordinarily low microbial count might point to a disrupted or repressed gut microbiota, which could be related to its anemia.

MATERIALS AND METHODS

1. INDOLE TEST

1.1 MATERIALS –

The primary criterion for a good indole test medium is that it has enough tryptophan. Tryptone broth is frequently utilized, albeit many media fulfil this requirement.

- ✓ Making Tryptophan broth is a must.
  - 10.0 g of tryptone
  - 5.0 g of sodium chloride

The ingredients should be dissolved in 1000 millilitre of sterile water at pH 7.4. Give each tube 4 ml. Autoclave the tubes for 15 minutes at 121°C under 15 psi pressure after capping them. Keep the tubes in the fridge at 4 to 10°C.

- ✓ Kovács reagent preparation
  - 150.0 mL of reagent grade amyl or isoamyl alcohol (butyl alcohol may be used instead)
  - 10.0 g of para-dimethylaminobenzaldehyde (DMAB)
  - 50.0 mL of concentrated HCl

To dissolve DMAB in the alcohol, you may need to apply a little heat to help the aldehyde dissolve. Gradually introduce the acid to the aldehyde-alcohol mixture. The remedy is only stable for a little period and should be a light yellow hue. The market also sells Kovács reagent. There were 23 strains in total, plus one positive control *E.coli*.

- ✓ Making of 75 mL Tryptophan broth.
  - 0.75 g of tryptone used
  - 0.375 g of NaCl used
  - 75 mL distilled water

For this experiment, the Kovács reagent from the market was used.

1.2 METHODS–

1. Each test tubes had 3 mL of tryptophan broth.
2. Little bit of a pure culture was introduced using an inoculating loop into the tryptone broth test tube.
3. Incubation temperature between 33°C and 37°C for 24 to 48 hours was maintained.
4. 5 drops of Kovács reagent was added directly to the tube to check for indole synthesis.

The formation of cherry-red ring in the reagent layer above the media suggests a positive indole test. The reagent layer will be yellow or somewhat hazy if a culture is indole negative.

2. METHYL RED TEST

2.1 MATERIALS –

- ✓ Inoculation loop
- ✓ Methyl red (MR)
- ✓ Making Buffered Glucose Broth for 1000 mL dH2O
    - Peptone – 10 g
    - NaCl – 5 grams
    - Disodium hydrogen phosphate – 3.5 g
    - Potassium dihydrogen phosphate – 1.5 g
    - pH 7.2

Methyl red indicator: 0.1 MR dissolved in 200 ml of 95% ethanol.

There were 23 strains for MR test in total, plus one positive control *E.coli*, for MR and one positive control for VP test.

- ✓ Making of 130 mL Buffered Glucose broth (preparing for both MR and VP test).
    - Peptone – 1.3 g
    - NaCl – 0.65 grams
    - Disodium hydrogen phosphate – 0.455 g
    - Potassium dihydrogen phosphate – 0.195 g
    - Distilled water – 130 mL

2.2 METHODS –

1. Buffered Glucose Broth is prepared and autoclaved.
2. The test tubes are filled with 2.5 mL autoclaved media.
3. In separate test tubes containing media, each organism is inoculated.
4. The test tubes are correctly labeled and kept at 37°C for 24–48 hours.
5. Methyl red 5 to 6 drops are put into the medium, and the results noted.

3. VOGES PROSKAUER TEST

3.1 MATERIALS –

- ✓ Making Buffered Glucose Broth for 1000 mL dH2O
  - Peptone – 10 g
  - NaCl – 5 grams
  - Disodium hydrogen phosphate – 3.5 g
  - Potassium dihydrogen phosphate – 1.5 g
  - pH 7.2

- ✓ Reagents
  - VP Reagent A: Barritt's Reagent A
    - Alpha Naphthol (5%): $5g$
    - Absolute Ethanol: $1000ml$
  - VP Reagent B: Barritt's Reagent B
    - KOH: $400g$
    - Deionized Water: $1000ml$
- ✓ Making of 130 mL Buffered Glucose broth (preparing for both MR and VP test).
  - Peptone – 1.3 g
  - NaCl – 0.65 grams
  - Disodium hydrogen phosphate – 0.455 g
  - Potassium dihydrogen phosphate – 0.195 g
  - Distilled water – 130 mL

3.2 METHODS –

1. Around 2.5-3 mL media is poured into test tubes.
2. Using a sterile inoculation loop, the different organisms are inoculated into the test tubes containing the media.
3. It is kept at 37°C for 24–48 hours.
4. The next day, the test tube is gently shaken after the addition of just a few drops of the two reagents: 6 drops of Barritt's reagent A, which is 5% α-napthol in absolute alcohol, and 2 drops of Barritt's reagent B, which is 40% KOH.

4. CITRATE UTILIZATION TEST

4.1 MATERIALS –

- ✓ Simmons Citrate Agar:
  - Sodium Chloride (NaCl) – 5.0g
  - Sodium Citrate (dehydrate) – 2.0g
  - Ammonium Dihydrogen Phosphate – 1.0g
  - Dipotassium Phosphate – 1.0g
  - Magnesium Sulfate (heptahydrate) – 0.2g
  - Bromothymol Blue – 0.08g
  - Agar – 15g

Deionized water = 1000 ml

Final pH 6.9 +/- 0.2 at 25°C.

There are 23 strains in total, plus one Citrate positive control.

- ✓ Making 130 mL Simmons Citrate Agar:
  - Sodium Chloride (NaCl) – 0.65g
  - Sodium Citrate (dehydrate) – 0.26g
  - Ammonium Dihydrogen Phosphate – 0.13g
  - Dipotassium Phosphate – 0.13g
  - Magnesium Sulfate (heptahydrate) – 0.026g
  - Bromothymol Blue – 0.0104g
  - Agar – 1.95g
  - Distilled water – 130mL

4.2 METHODS –

1. In distilled water, the salts are dissolved.
2. Bromothymol blue and agar are introduced.

3. Till the agar melted, it was gently heated while stirring.

4. Petri dishes can use the medium as a plate medium, whereas test tubes can utilize it as slopes. In both cases, the medium's surface is softly inoculated by streaking.

5. Deep green media is dispensed into 16-mm tubes at a rate of 4.0 to 5.0 ml per tube.

6. Autoclaving is performed for 15 minutes at 121°C under 15 psi pressure.

7. The tubes are cooled at an angle (with a lengthy slope and a low butt).

5. UREASE TEST

5.1 MATERIALS –

- ✓ Christensen Urea Agar for 1000 mL distilled water
    - Peptone – 1 g
    - Dextrose – 1 g
    - Sodium chloride – 5 g
    - Potassium phosphate, monobasic – 2 g
    - Urea – 20 g
    - Phenol red – 0.012 g
    - Agar – 15 to 20 g

The first six components were dissolved in 100 mL of distilled water. Autoclaving of the agar after it has been boiled and thoroughly dissolved in 900 ml of distilled water at 121°C and 15 psi for 15 minutes, was done. In a sterile manner, 100 ml of filter-sterilized urea base was added to the chilled agar solution, mixing well. 5 ml of media poured into each sterile tube (13 x 100 mm) and tilt the tubes throughout cooling until they are firm. Prepared media was refrigerated at 4 to 8°C. No reheating of the medium after it has been prepared since the urea will break down if reheated.

There are 23 strains in total, plus one Urease *Enterobacter* positive control.

- ✓ Christensen Urea Agar for 130 mL
    - Peptone – 0.13 g
    - Dextrose – 0.13 g
    - Sodium chloride – 0.65 g
    - Potassium phosphate, monobasic – 0.26 g
    - Urea – 2.6 g
    - Phenol red – 0.00156 g
    - Agar – 1.95g
    - Distilled water – 130mL

So, first six components were dissolved in 13 mL of distilled water. Autoclaving of the agar after it has been boiled and thoroughly dissolved in 117 ml of distilled water at 121°C and 15

psi for 15 minutes, was done. In a sterile manner, 13 ml of filter-sterilized urea base was added to the chilled agar solution, mixing well. 5 ml of media poured into each sterile tube (13 x 100 mm) and tilt the tubes throughout cooling until they are firm.

5.2 METHODS –

1. The entire slant surface was streaked, from pure culture.
2. Place tubes with loose lids in a 35°C incubator.
3. Examine the slope for a shift in color at 6 hours, 24 hours, and every day for a period of six days.

6. TRIPLE SUGAR IRON (TSI) TEST

6.1 MATERIALS –

- ✓ TSI agar preparation
  - Beef extract – 3.0g
  - Yeast extract – 3.0g
  - Peptone – 20g
  - Glucose – 1.0 g
  - Lactose – 10.0 g
  - Sucrose – 10.0 g
  - Ferrous sulphate – 0.2 g
  - NaCl – 5 g
  - Sodium thiosulfate – 0.3 g
  - Phenol red – 0.024 g
  - Agar – 13.0 g
  - Distilled water – 1000 mL

Combine ingredients, and adjust the pH to 7.3. Autoclaving at 121°C for 15 min. Cooling in a slanted position.

There were 14 strains in total, with one *E. coli* positive control.

- ✓ 100 mL TSI agar preparation
  - Beef extract – 0.3g

- Yeast extract – 0.3g
- Peptone – 2.0g
- Glucose – 0.1 g
- Lactose – 1.0 g
- Sucrose – 1.0 g
- Ferrous sulfate – 0.02 g
- NaCl – 0.5 g
- Sodium thiosulfate – 0.03 g
- Phenol red – 0.0024 g
- Agar – 1.3 g

Distilled water – 100 mL

6.2 METHODS –

1. An isolated colony picked up using a straight inoculating needle.
2. To inoculate the TSI slant, sticking the butt of the needle down to the bottom done, then the needle was pulled back, and then the surface of the slant was streaked.
3. Loose closing to allow air access was done. Incubation at 37°C for 24 hours. Then results were read. The reactions can produce three different kinds of data.

- Sugar fermentations: yellow acidic butt, red basic slant, glucose fermented but sucrose or lactose haven't. Lactose and/or sucrose fermentation, yellow acidic butt, and yellow acidic slant. Red alk. if no fermentation of glucose, lactose, or sucrose.
- Gas production, which is evidenced by bubbles in the buttocks. The agar could break or be pushed up by a lot of gas.
- Hydrogen sulfide generation - The formation of black ferrous sulfide from the interaction of H_2S with the ferrous ammonium sulfate causes the butt to turn black, indicating that hydrogen sulfide is produced from thiosulfate. The bacteria's capacity to create hydrogen sulfide (H_2S) from sodium thiosulfate is shown by the black precipitate. Since H_2S is colorless, ferric ammonium citrate is employed as an indicator, which causes the production of insoluble ferrous sulfide. An acidic environment is necessary for the creation of H_2S, and although the black precipitate makes it impossible to see a yellow butt, the butt is still acidic. Acid over acid (A/A) and H_2S positive are the ways the outcomes will be documented.

7. CATALASE TEST

7.1 MATERIALS –

3% hydrogen peroxide is used, which is readily available for purchase, for regular aerobic testing. Hydrogen peroxide is kept in a dark bottle in the refrigerator. A 15% H_2O_2 solution is required to identify anaerobic bacteria. For 23 strains and one positive *S.aureus*, the test is performed.

7.2 METHODS –

1. A clean glass slide is divided into several sections, one for the experiment and one for the control.
2. A tiny drop of regular saline solution was put on each area. A little amount of bacterial culture with a sterile inoculating loop was taken and spread over the saline drop to form a uniform suspension.
3. Using a Pasteur pipette, one drop of 3% hydrogen peroxide was put to the test smear.
4. The slide was checked right away for the development of gas bubbles.

8. COAGULASE TEST

8.1 MATERIALS–

As lyophilized citrated or EDTA-treated rabbit plasma, commercially available coagulase plasma is purchased from a provider and rehydrated with sterile distilled water just before usage. As previously stated, EDTA therapy is preferable. The manufacturer will specify the quantity of water required for rehydration. Since plasma is inactivated by cycles of thawing and refreezing, frozen aliquots should be kept in a freezer that is not frost-free.

- Overnight plate culture of the organism to be tested on noninhibitory agar, utilizing a microscope slide
- Control organisms, typically comprised *S. aureus* (positive control).
- Physiological saline or water prepared with 0.09 g of NaCl in 10 mL of distilled water, and autoclaved.
- Unsterilized dropper

• Reconstituted plasma coagulase
• Sterile pipette

8.2 METHODS –

1. A tiny drop (10-20 microlitre) of water or saline was put into the slide using the nonsterile dropper. The water or saline serves as a control to make sure that the strain readily emulsifies rather than auto-agglutinating and clumping with itself.
2. A tiny drop of reconstituted plasma was put into the circle at the other end of the slide using the sterile dropper.
3. Using the loop or applicator stick, cells from one colony were taken and emulsified in the water (or saline) and then in the drop of plasma. The sequence is crucial to prevent trace quantities of plasma from being transferred into the control drop.
4. Clumping within 10 seconds of introducing the bacterial cells to the plasma is an indicator of coagulase positive organism. If the slide is gently rocked in a figure 8 manner, the clumping will become more apparent.

9. GRAM STAINING

9.1 MATERIALS –

- ✓ Crystal Violet
- ✓ Methylene Blue
- ✓ Potassium Iodide
- ✓ Ethanol, 95%
- ✓ Acetone
- ✓ Ammonium oxalate
- ✓ Iodine
- ✓ Sodium bicarbonate
- ✓ Safranin O
- ✓ Bunsen Burner
- ✓ Microscope
- ✓ Slide
- ✓ Distilled water

9.2 METHODS-

✓ Making a slide smear.

1. To take a culture from a petri dish, a tiny amount of a colony from the petri dish was aseptically transfered onto the slide after adding a drop of water.
2. Using an inoculation loop, the culture is distributed to form an uniform, thin layer over a circle that is 1.5 cm in diameter (about the size of a dime).
3. A sterile forcep was used to hold the slide.

✓ Gram staining

1. Few drops of cv dye was put, and allowed to stand for 60 seconds.
2. With a stream of distilled water, the stain was washed away and any remaining stain. The aim of this procedure is to get rid of the stain, not the set culture.
3. Then iodine solution was put on the smear, using around 5 drops, or enough to coat the fixed culture, and allowed to sit for sixty seconds.
4. Washing of the the slides under running water was done, after pouring out the iodine solution to get rid of any extra water on the surface.
5. Few drops of alcohol acetone decolorizer was put to the solution so that it drips down the slide. After thirty seconds, it was rinsed with water. When the solvent is no longer colored as it runs over the slide, washing was stopped.
6. After 20 seconds, counterstaining with 5 drops of the Safranin solution was done.
7. Rinsing of the red Safranin solution was done with water. Bibulous paper was used to absorb extra water.
8. Immediate rinsing off any spilled stain with lots of water was done, to prevent lasting stains on the sink, lab bench, or glassware.
9. Observation of the completed slide under a microscope was done.

10. KIRBY BAUER DISK DIFFUSION SUSCEPTIBILITY TEST

10.1 MATERIALS –

✓ Nutrient Agar – 28.0 g in 1000 mL distilled water
✓ Nutrient broth – 13.0 g in 1000 mL distilled water
✓ Petri plates

- ✓ Antibiotic Discs (In our experiment we used Penicillin, Tetracycline, Streptomycin and Chloramphenicol)
- ✓ Forceps
- ✓ Inoculum
- ✓ Metric ruler or Calipers
- ✓ Preparation of Nutrient agar plates
    - Nutrient agar required for 300 mL of distilled water – 8.4 g
    - After autoclaving at 15psi for 15 minutes at 121℃, the nutrient agar was poured in the petri dishes.
    - After it solidified, it was put in the incubator overnight, at 37℃.
- ✓ Preparation of Nutrient agar broth
    - NB for 50 mL of dH2O – 0.65 g. autoclave.

10.2 METHODS –

1. The organisms must be in the log phase for the data to be accurate. In order to ensure that the test organism's subcultures are maintained in nutrient broth medium, they must be created the day before. Once the O.D of the culture measured at 0.5, the culture can be spread on the nutrient agar plates.

2. Making the NA plates: The plates should have a thickness of 4 mm (roughly 20-25 ml of liquid agar for 100-mm plates). The agar medium should be exactly 4 mm thick since the zone of inhibition will be smaller the thicker it is. The pH of the agar should be between 7.2 and 7.4 at ambient temperature.

3. Using a spreader, 100 microlitre of bacterial culture was introduced onto the agar plates and evenly distributed. Then I let it wait for 5-10 minutes.

4. The antibiotic discs were placed on the agar with a forcep.

5. The plates are positioned so that their zone of inhibition does not overlap.

6. After positioning all of the discs, the plates are incubated for 16 to 18 hours at 37°C.

10. MACCONKEY AGAR PLATES

11.1 MATERIALS –

- ✓ MacConkey Agar – 51.53 g in 1000 mL distilled water
- ✓ Petri plates

- ✓ Preparation of MacConkey agar plate
    - MacConkey Agar for 20 mL dH2O – 1.0306 g
    - After autoclaving at 15psi for 15 minutes at 121℃, the media was poured in the petri dish.
    - After it solidified, it was put in the incubator overnight, at 37℃.

11.2 METHODS –

1. A plate was divided into four segments.
2. The Gram-negative rods, along with a control (*E.coli*), were streaked on the MacConkey agar plate, using sterile inoculating loop.
3. The plate was kept in the incubator for 24-48 hours, at 37℃.

RESUILTS AND DISCUSSIONS

- *Table1.: The strains obtained after spread plating 100 microlitre of the bacterial extracts obtained from ileum, colon and feces of the mice, and incubated overnight at 37°C, are named as follows –*

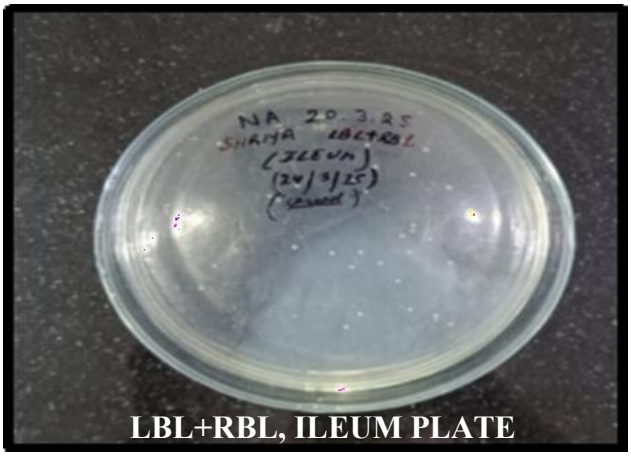
Mouse name	No. of strains	Name of strains	Appearance to naked eye
1. LBL+RBL	Ileum - 1	IL, ILEUM	White, small
	Colon - 0	Not any	Not any
2. B+T+LBL	Ileum - 4	IWB	White, big
		IWM	White, medium
		IWS	White, small
		IY	Yellow
	Colon - 4	CWB	White, big
		CWM	White, medium
		CWS	White, small
		CY	Yellow
3. B+T+RFL	Ileum - 4	IWB	White, big
		IO	Orange
		IWS	White, small
		IY	Yellow
	Colon - 4	CWB	White, big
		CO	Orange
		CWS	White, small
		CY	Yellow
4. LBL+RBL (control)	Ileum - 2	IWB	White, big
		IWS	White, small
	Colon - 2	CWB	White, big

		CWS	White, small
	Feces - 2	FWB	White, big
		FWS	White, small

❖ *Table2.: The colony morphology are as follows –*

Mouse name	No. of strains	Name of strains	Shape	Elevation	Margin
1. LBL+RBL	Ileum - 1	IL, ILEUM	Circular, round	Convex	Entire
	Colon - 0	Not any	Not any	Not any	Not any
2. B+T+LBL	Ileum - 4	IWB	Circular, round	Flat	Undulate
		IWM	Circular, round	Flat	Entire
		IWS	Circular, round	Convex	Entire
		IY	Circular, round	Flat	Undulate
	Colon - 4	CWB	Circular, round	Flat	Undulate
		CWM	Circular, round	Flat	Entire
		CWS	Circular, round	Flat	Entire
		CY	Circular, round	Flat	Undulate
3. B+T+RFL	Ileum - 4	IWB	Circular, round	Flat	Entire
		IO	Circular, round	Flat	Entire
		IWS	Circular, round	Flat	Entire
		IY	Circular, round	Flat	Entire
	Colon - 4	CWB	Circular, round	Flat	Entire
		CO	Circular, round	Flat	Entire
		CWS	Circular, round	Flat	Entire
		CY	Circular, round	Flat	Entire

4. LBL+RBL (control)	Ileum - 2	IWB	Circular, round	Convex	Entire
		IWS	Circular, round	Flat	Entire
	Colon - 2	CWB	Circular, round	Flat	Entire
		CWS	Circular, round	Flat	Entire
	Feces - 2	FWB	Circular, round	Flat	Entire
		FWS	Circular, round	Flat	Entire



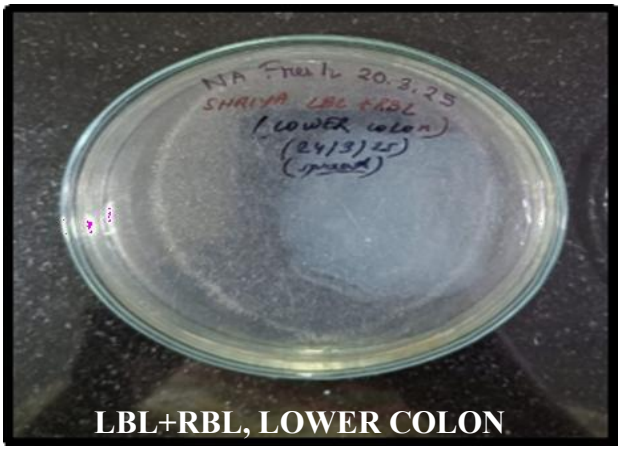


Fig1&2.: Spread plating of 10^{-3} dilution of the bacterial extract from Gut of anemic mouse LBL+RBL

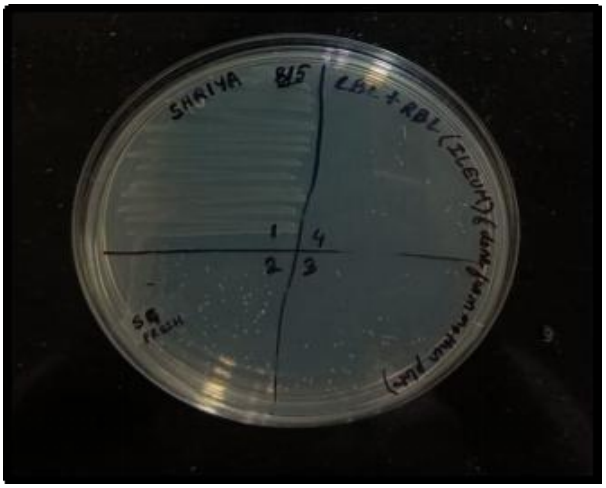


Fig3.: Quadrant streaking done for Pure colony isolation of bacterial colonies in anemic mouse's ileum LBL+RBL

Fig4.: Spread plating of 10^{-3} dilution of the bacterial extract from ileum and colon of anemic mouse B+T+LBL

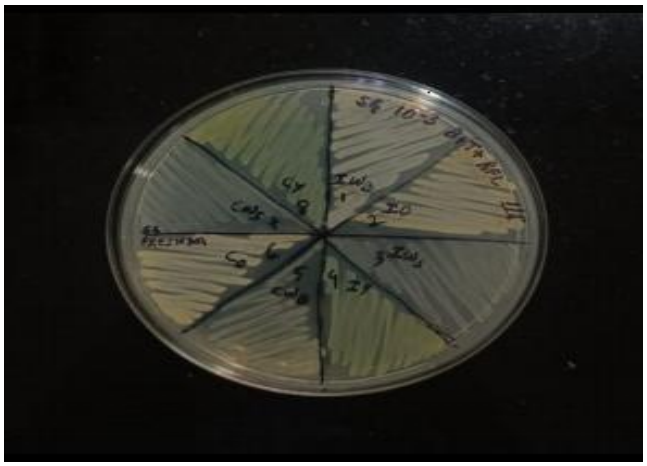


Fig5.: Sector streaking done for Pure colony isolation of bacterial colonies in anemic mouse B+T+LBL

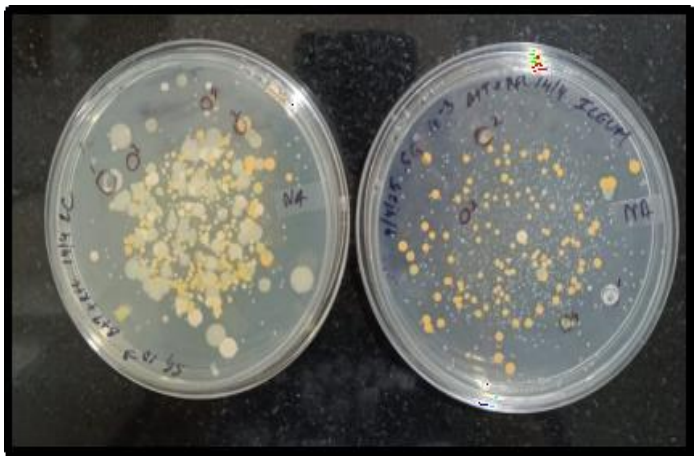


Fig6.: Spread plating of 10^-3 dilution of the bacterial extract from ileum and colon of anemic mouse B+T+RFL

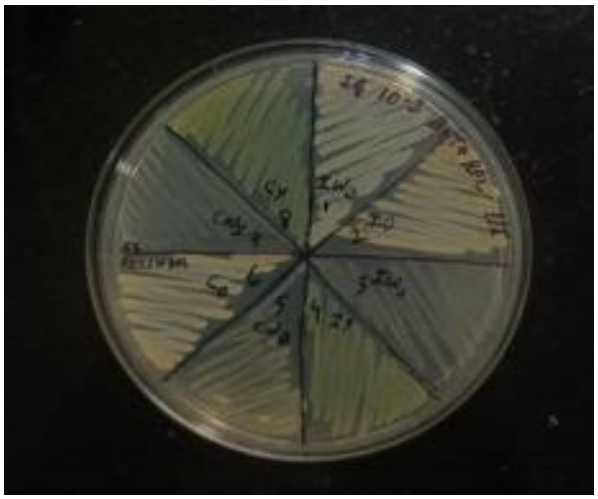


Fig7.: Sector streaking done for Pure colony isolation of bacterial colonies in anemic mouse B+T+RFL

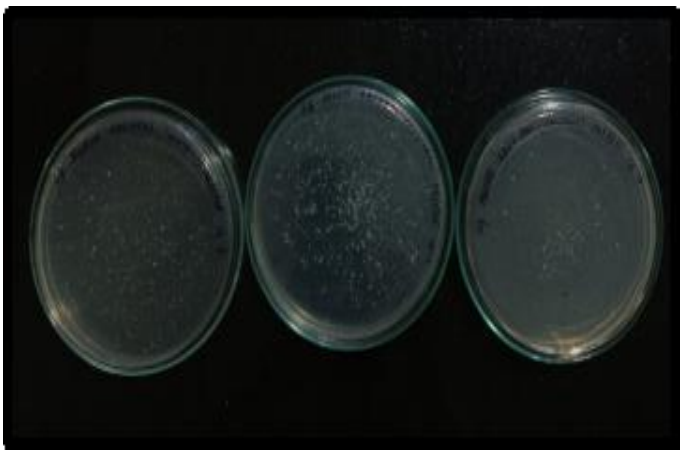


Fig8.: Spread plating of 10^-3 dilution of the bacterial extract from ileum, colon and feces of undiseased normal mouse LBL+RBL (CONTROL)

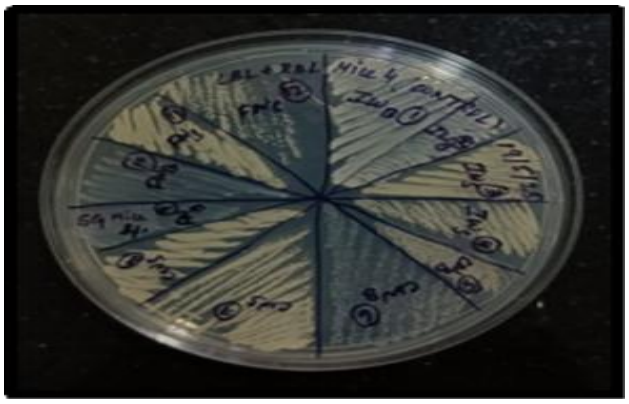


Fig9.: Sector streaking done for Pure colony isolation of bacterial colonies in undiseased normal mouse LBL+RBL

The colony morphology of the 23 bacterial strains isolated and cultured on nutrient agar plates revealed consistent and notable characteristics. All observed colonies exhibited a circular, round shape, indicating a uniform growth pattern across the different strains. The colony margins were predominantly entire, with some strains showing undulate edges. Color-wise, most of the bacterial colonies were white, while a smaller number displayed yellow or orange pigmentation. These morphological features—shape, elevation, margin—provide preliminary insight into the diversity and identity of the bacterial strains and serve as an important basis for further microbiological characterization.

Interestingly, no bacterial colonies were observed from the colon sample of Mouse LBL+RBL, which was an anemic mouse. This absence of growth may be associated with altered gut microbiota or reduced bacterial load due to the disease condition. The first three mice, LBL+RBL, B+T+LBL, B+T+RFL, were anemic, while Mouse LBL+RBL (CONTROL) served as a non-disease control. This observation may point to a possible impact of anemia on the composition or viability of gut microbiota.

❖ *Table3.: Indole, Methyl red, Voges Proskauer, Citrate (IMViC) results and discussion*

Mouse name	No. of strains	Name of strains	Indole	MR	VP	Citrate
1. LBL+RBL	Ileum - 1	IL, ILEUM	–	+	–	–
	Colon - 0	Not any	Not any	Not any	Not any	Not any
2. B+T+LBL	Ileum - 4	IWB	+	–	–	–
		IWM	+	+	+	+
		IWS	–	+	+	–
		IY	–	+	+	–
	Colon - 4	CWB	–	+	–	–
		CWM	–	–	+	–
		CWS	–	+	+	–
		CY	–	–	–	–
3. B+T+RFL	Ileum - 4	IWB	–	+	+	–

		IO	–	+	+	–
		IWS	–	+	+	–
		IY	–	–	+	–
	Colon - 4	CWB	–	–	–	+
		CO	–	+	+	–
		CWS	–	+	+	–
		CY	–	–	+	–
						–
4. LBL+RBL (control)	Ileum - 2	IWB	–	+	–	–
		IWS	–	–	–	–
	Colon - 2	CWB	–	–	+	–
		CWS	–	–	+	–
	Feces - 2	FWB	–	–	–	–
		FWS	–	+	–	–

Fig10.: INDOLE +VE CONTROL – *E. coli*

Fig11.: ILEUM OF LBL+RBL - MR TEST RESULT

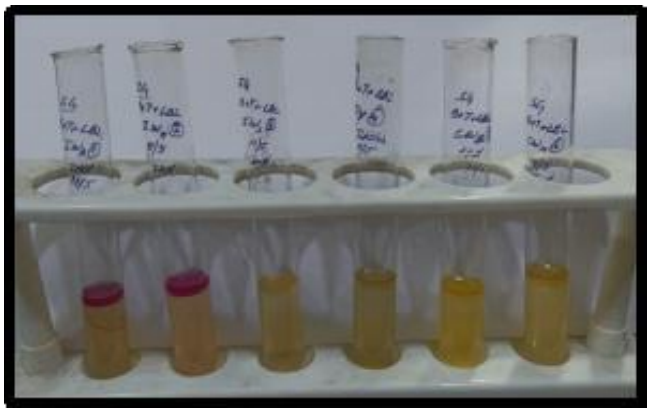


Fig12&13.: ILEUM AND COLON OF B+T+LBL - INDOLE TEST RESULT

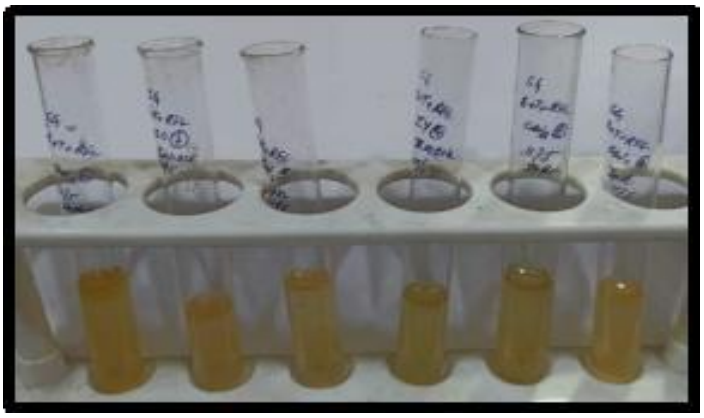


Fig14&15.: ILEUM AND COLON OF B+T+RFL - INDOLE TEST RESULT

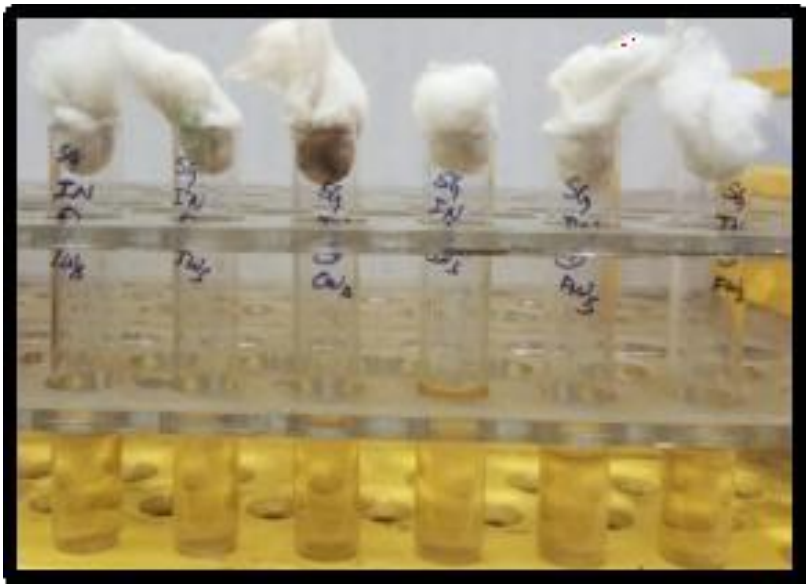


Fig16.: ILEUM, COLON, FECES OF LBL+RBL (CONTROL) VP TEST RESULTS

Fig17.: MR +VE CONTROL – *E. coli*

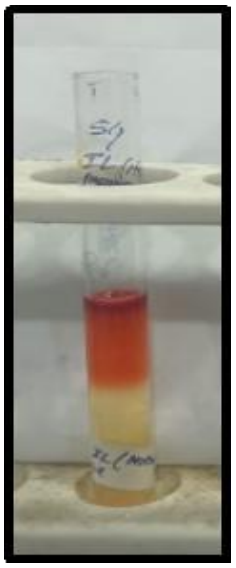


Fig18.: ILEUM OF LBL+RBL - MR TEST RESULT

Fig19&20.: ILEUM AND COLON OF B+T+LBL - MR TEST RESULT

Fig21.: ILEUM, COLON, FECES OF LBL+RBL (CONTROL) MR TEST RESULTS

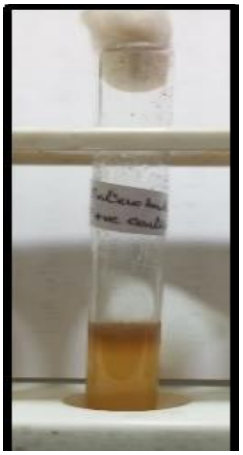


Fig22.: VP +VE CONTROL – *Enterobacter*

Fig23.: ILEUM OF LBL+RBL VP TEST RESULT

Fig24&25.: ILEUM AND COLON OF B+T+LBL - VP TEST RESULT

Fig26&27.: ILEUM AND COLON OF B+T+RFL - VP TEST RESULT

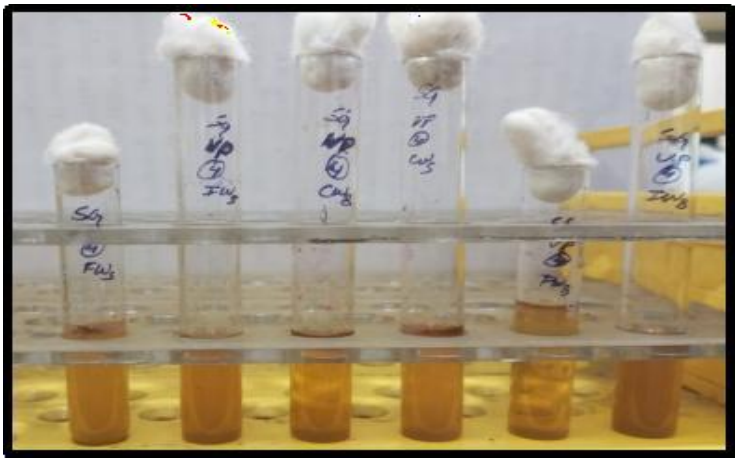


Fig28.: ILEUM, COLON, FECES OF LBL+RBL (CONTROL) VP TEST RESULTS

Fig29.: CITRATE +VE CONTROL - *Enterobacter*

Fig30.: ILEUM OF LBL+RBL CITRATE TEST RESULT

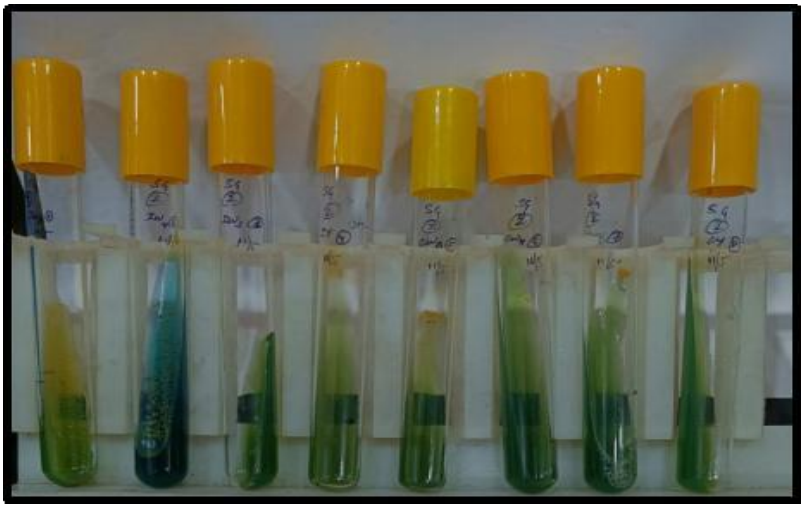


Fig31.: ILEUM AND COLON OF B+T+LBL - CITRATE TEST RESULT

Fig32.: ILEUM AND COLON OF B+T+RFL - CITRATE TEST RESULT

Fig33.: ILEUM, COLON, FECES OF LBL+RBL (CONTROL) CITRATE TEST RESULTS

In standard *Enterobacteriaceae* differentiation: MR-positive indicates mixed acid fermentation, seen in *E. coli, Shigella*, and others. Rarely are both MR and VP strongly positive in the same strain.

So if most of the isolates are showing both MR and VP positivity, this suggests one or more of the following:

- Mixed fermentation capabilities, possibly pointing toward intermediate fermenters or environmental enteric bacteria with variable fermentation pathways.

- Non-Enterobacteriaceae strains that may exhibit both MR and VP positivity due to unique metabolic traits.
- Slight cross-reactivity or borderline results—sometimes, weak VP or MR reactions may give a false sense of dual-positivity, depending on incubation and interpretation timing.

Most strains show citrate negative. This indicates that most of the strains cannot utilize citrate as the sole carbon source, ruling out typical *Enterobacter* or *Klebsiella*, which are usually citrate positive. The bacterial strains isolated from mice gut microbiota may predominantly consist of non-pathogenic or commensal bacteria that: Carry out mixed acid fermentation (MR+), have limited ability to use citrate (Citrate−), show some acetoin production (VP+), do not produce indole (Indole−), likely due to lack of tryptophanase enzyme.

This is unusual because most organisms do not show both MR and VP positive. That typically suggests metabolic flexibility—i.e., the bacteria can carry out both mixed acid and butylene glycol fermentation to some degree.

Possible bacteria can be environmental *Escherichia coli* as some *E. coli* strains, especially commensal gut isolates, may show weak VP positivity under certain conditions. They are often MR+, Citrate−, and some may be Indole−. It can also be a less common *Enterobacteriaceae* or closely related genera like *Hafnia* or *Pantoea* may exhibit mixed IMViC results and are found in gut flora or the environment.

❖ *Table4.: Urease, Catalase, Coagulase results and discussions*

Mouse name	No. of strains	Name of strains	Urease	Catalase	Coagulase
1. LBL+RBL	Ileum - 1	IL, ILEUM	–	–	–
	Colon - 0	Not any	Not any	Not any	Not any
2. B+T+LBL	Ileum - 4	IWB	–	–	+
		IWM	–	–	+
		IWS	+	–	–
		IY	+	–	+
	Colon - 4	CWB	+	–	+

		CWM	–	–	–
		CWS	+	–	–
		CY	+	–	–
3. B+T+RFL	Ileum - 4	IWB	+	–	+
		IO	+	–	+
		IWS	+	–	–
		IY	–	–	–
	Colon - 4	CWB	–	–	–
		CO	+	–	–
		CWS	+	–	–
		CY	–	–	–
4. LBL+RBL (control)	Ileum - 2	IWB	+	–	–
		IWS	+	–	+
	Colon - 2	CWB	+	–	–
		CWS	+	–	+
	Feces - 2	FWB	–	–	–
		FWS	+	–	+

Fig34.: UREASE +VE CONTROL – *Enterobacter*

Fig35.: ILEUM OF LBL+RBL UREASE TEST RESULT

Fig36.: ILEUM AND COLON OF B+T+LBL - UREASE TEST RESULT

Fig37.: ILEUM AND COLON OF B+T+RFL - UREASE TEST RESULT

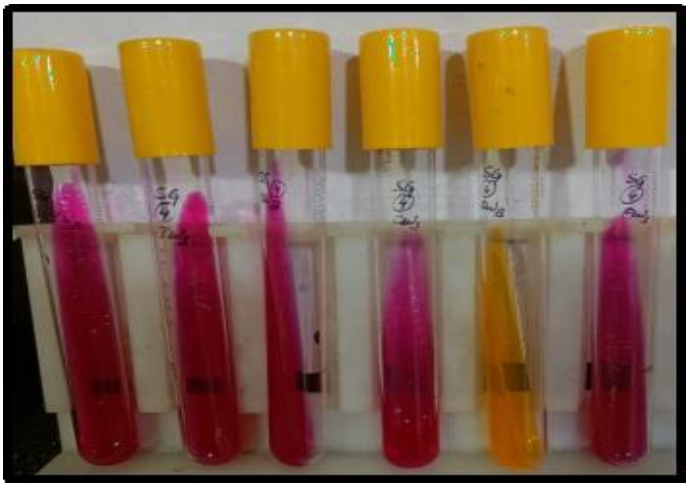


Fig38.: ILEUM, COLON, FECES OF LBL+RBL (CONTROL) UREASE TEST RESULTS

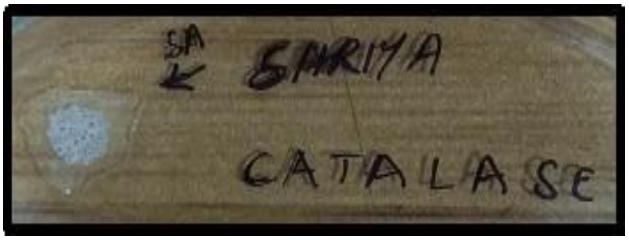


Fig39.: +VE CATALASE CONTROL – *Staphylococcus aureus*

Fig40.: ILEUM OF LBL+RBL - CATALASE TEST RESULT

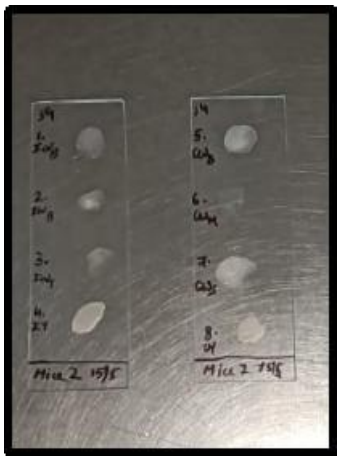


Fig41.: ILEUM AND COLON OF B+T+LBL - CATALASE TEST RESULT

Fig42.: ILEUM AND COLON OF B+T+RFL - CATALASE TEST RESULT

Fig43.: ILEUM, COLON, FECES OF LBL+RBL (CONTROL) CATALASE TEST RESULTS

Fig44.: +VE COAGULASE CONTROL – *Staphylococcus*

Fig45.: ILEUM OF LBL+RBL - COAGULASE TEST RESULT

Fig46.: ILEUM, COLON, FECES OF LBL+RBL (CONTROL) COAGULASE TEST RESULTS

Fig47.: ILEUM AND COLON OF B+T+LBL - COAGULASE TEST RESULT

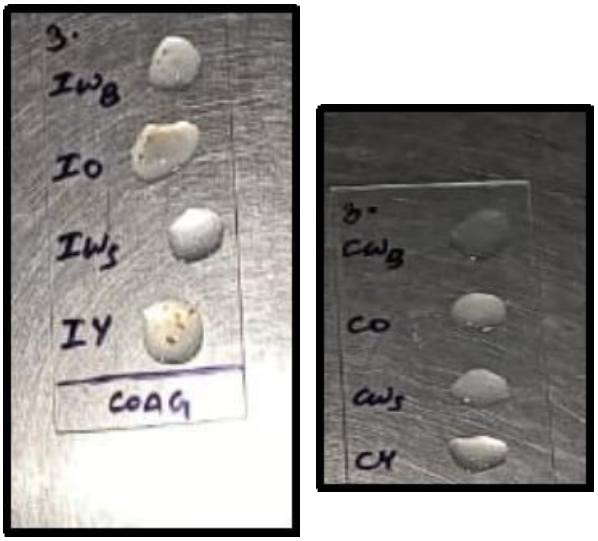


Fig48.: ILEUM AND COLON OF B+T+RFL - COAGULASE TEST

Most strains are urease positive. Urease hydrolyzes urea into ammonia and CO_2. Most strains show coagulase negative. All Strains are catalase negative. Catalase-negative bacteria are typically *Streptococci, Enterococci*, and some anaerobes. But *Staphylococcus* spp. are catalase positive, and many Gram-negative rods too. Since the strains are catalase negative, these are not *Staphylococci*, so this result further confirms that.

The most striking and narrowing trait here is Catalase Negative in all strains, which significantly narrows it down. When combined with: Urease Positive, Coagulase Negative, it can most likely be a Gram-positive cocci, specifically in the genera:

- *Enterococcus* spp. – Catalase negative, some strains are urease positive, non-hemolytic or γ-hemolytic usually, found in gut flora, especially in humans and animals, often coagulase negative, associated with both health and opportunistic infections.
- *Streptococcus* spp. – Catalase negative, typically urease negative, but some environmental or commensal species may show variable urease activity.
- Anaerobic cocci (e.g*., Peptostreptococcus*) – Catalase negative, may be urease positive, commensals of the gut and mouth.

The most likely candidates are *Enterococcus* species, possibly *Enterococcus faecalis* or *Enterococcus durans*, which are common gut commensals and fit this biochemical profile. These are often facultative anaerobes, survive well in nutrient agar, and are known to show urease activity in some strains.

❖ *Table5.: Triple sugar iron (TSI) results and discussions*

Mouse name	No. of strains	Name of strains	TSI
1. LBL+RBL	Ileum - 1	IL, ILEUM	–
	Colon - 0	Not any	Not any
2. B+T+LBL	Ileum - 4	IWB	+++
		IY	+++
	Colon - 4	CY	+++

3. B+T+RFL	Ileum - 4	IO	++
		IY	–
	Colon - 4	CO	–
		CY	+
4. LBL+RBL (control)	Ileum - 2	IWB	–
		IWS	+
	Colon - 2	CWB	–
		CWS	–
	Feces - 2	FWB	++
		FWS	+++

Fig49.: +VE CONTROL FOR TSI – *E.coli*

Fig50.: TSI RESULTS OF LBL+RBL, B+T+LBL, B+T+RFL

Fig51.: TSI RESULTS OF LBL+RBL (CONTROL)

Triple Sugar Iron (TSI) test is a powerful tool to evaluate carbohydrate fermentation (glucose, lactose, sucrose) and hydrogen sulfide (H_2S) production in Gram-negative enteric bacteria. 14 out of 23 strains were tested. 8 are positive, could be *Escherichia coli, Enterobacter* spp.

A positive TSI test typically refers to: Acid production (seen as yellow color changes), gas production (bubbles/cracks in the agar), hydrogen sulfide (H_2S) production (black precipitate). So, a positive result usually means that the bacteria can: Ferment glucose (and possibly lactose/sucrose), possibly produce gas or H_2S, likely belong to *Enterobacteriaceae* or related fermentative Gram-negative rods.

A negative TSI test means: No sugar fermentation (no color change or slant/butt stays red), no acid/gas/H_2S production. This suggests that the bacteria do not ferment glucose, lactose, or sucrose, may be non-enteric, could be Gram-positive cocci like *Streptococcus* or *Enterococcus* (they often don't grow well or react clearly on TSI slants), or non-fermenting Gram-negative rods like *Pseudomonas.*

These 6 strains could be: *Enterococcus* spp., *Streptococcus* spp.

The 8 TSI-positive strains likely include fermentative Gram-negative enteric bacteria, most plausibly from the Enterobacteriaceae family (*E. coli, Klebsiella*, etc.).

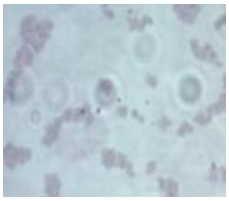
The 6 TSI-negative strains may represent Gram-positive cocci like *Enterococcus* spp. or non-fermenting bacteria that do not utilize the sugars in the TSI medium.

❖ *Table6.: Gram staining results and discussions*

Mouse name	No. of strains	Name of strains	Gram staining
1. LBL+RBL	Ileum - 1	IL, ILEUM	+, cocci
	Colon - 0	Not any	Not any
2. B+T+LBL	Ileum - 4	IWB	–, bacilli
		IWM	–, bacilli
		IWS	+, bacilli
		IY	+, cocci
	Colon - 4	CWB	–, cocci
		CWM	–, bacilli

		CWS	+, cocci
		CY	–, cocci
❖ B+T+RFL	Ileum - 4	IWB	+, cocci
		IO	–, cocci
		IWS	–, cocci
		IY	+, cocci
	Colon - 4	CWB	–, bacilli
		CO	+, cocci
		CWS	–, cocci
		CY	+, cocci
❖ LBL+RBL (control)	Ileum - 2	IWB	+, cocci
		IWS	+, cocci
	Colon - 2	CWB	–, cocci
		CWS	+, cocci
	Feces - 2	FWB	–, coccobacilli
		FWS	+, cocci

Fig52.: LBL+RBL ILEUM GRAM STAINING RESULTS UNDER 100X MAGNIFICATION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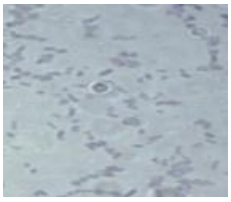
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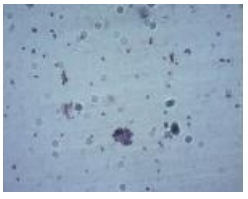
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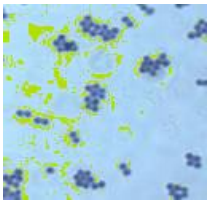
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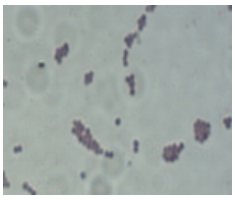
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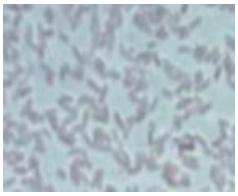
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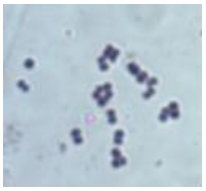
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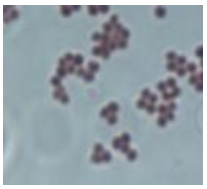
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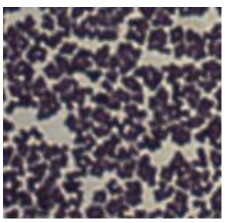
Fig53.: B+T+LBL ILEUM AND COLON GRAM STAINING RESULTS UNDER 100X MAGNIFICATION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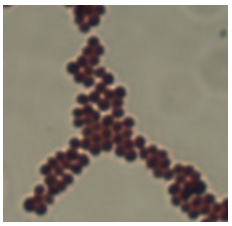
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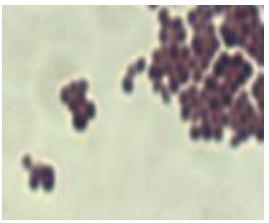
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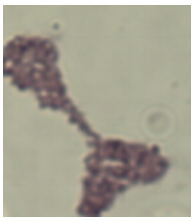
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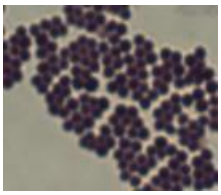
CWB

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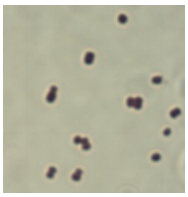
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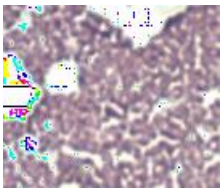
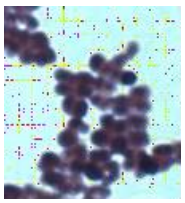

CY

Fig54.: B+T+RFL ILEUM AND COLON GRAM STAINING RESULTS UNDER 100X

IWB

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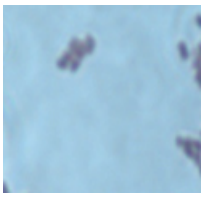
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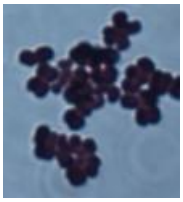
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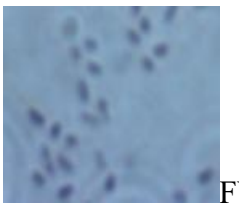
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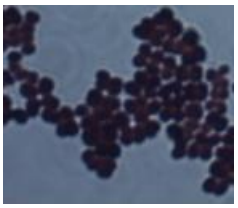
FWS

Fig55.: LBL+RBL ILEUM, COLON, FECES GRAM STAINING RESULTS UNDER 100X MAGNIFICATION

Most strains: Gram-positive cocci in clusters or tetrads

Some strains: Gram-negative cocci in clusters

1 strain: Gram-negative cocci in pairs

4 strains: Gram-negative bacilli

1. Gram-Positive Cocci in Clusters: These are likely *staphylococci* or similar genera. But you noted all were catalase-negative, which rules out *Staphylococcus*. Possibilities include: *Enterococcus* spp. (though often diplococci or chains, but variable). Since they're urease positive and catalase negative, they are most likely *Enterococcus* spp.
2. Gram-Negative Cocci in Clusters: Gram-negative cocci are uncommon; when present in clusters, possibilities include: *Moraxella* spp. However, clusters are not typical for these genera — this may be a staining or arrangement artifact. If catalase-negative: possibly misidentified coccoid rods, or fastidious bacteria. These could also be fragile Gram-negative rods that appeared coccoid—seen with *Haemophilus* or stressed cells.
3. Gram-Negative Bacilli: These could be *Enterobacteriaceae*, like E. coli, *Klebsiella*, *Enterobacter*, etc. TSI-positive strains probably fall into this group.
   These are non-classical enteric Gram-negatives, possibly stressed or slow-growing. That's why they may be catalase negative.
   Colony morphology, TSI, IMViC, and urease tests collectively point toward:
   Enterococcus (Gram+ cocci), *Enterobacteriaceae* (Gram− rods, from TSI+ strains)

❖ *Table7.: Kirby Bauer Disk Diffusion Susceptibility Test*

Mouse name	**No. of strains**	**Name of strains**	**Methycillin**	**Ampicillin**	**Chloramphenicol**	**Vancomycin**	**Tetracycline**
1. LBL+RBL	Ileum - 1	IL, ILEUM	R	R	R	S 1.0	R
	Colon - 0	Not any	Not any	Not any	Not any	Not any	Not any
2. B+T+LBL	Ileum - 4	IWB	R	S 0.5	S 1.5	S 0.5	S 1.0
		IWM	R	S 0.5	S 1.5	S 0.5	R
		IWS	R	R	R	S	S

						1.0	1.5
		IY	R	S 1.0	S 1.5	S 1.0	S 1.5
	Colon - 4	CWB	R	S 1.5	S 1.5	S 1.0	S 2.0
		CW M	R	R	S 1.5	S 1.0	S 1.0
		CWS	R	R	R	S 1.5	R
		CY	R	S 1.5	S 1.5	S 1.2	S 1.5
3.LBL+RBL (control)	Ileum - 2	IWB	R	S 1.0	S 1.5	S 1.5	S 0.5
		IWS	R	S 0.5	S 2.5	S 1.5	S 2.0
	Colon - 2	CWB	R	S 1.0	S 3.0	S 1.5	S 4.0
		CWS	S 0.5	S 1.0	S 3.0	S 1.0	S 3.0
	Feces - 2	FWB	R	S 1.0	S 1.5	S 1.0	S 2.5
		FWS	R	R	S 1.5	S 1.0	S 2.0

R – resistant; S – sensitive ; diameter of zone of inhibition given in cm.

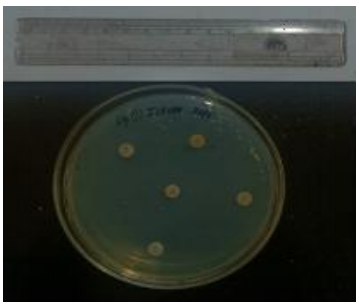

Fig56.: Kirby Bauer Disk Diffusion Susceptibility Test Results for Ileum of LBL+RBL

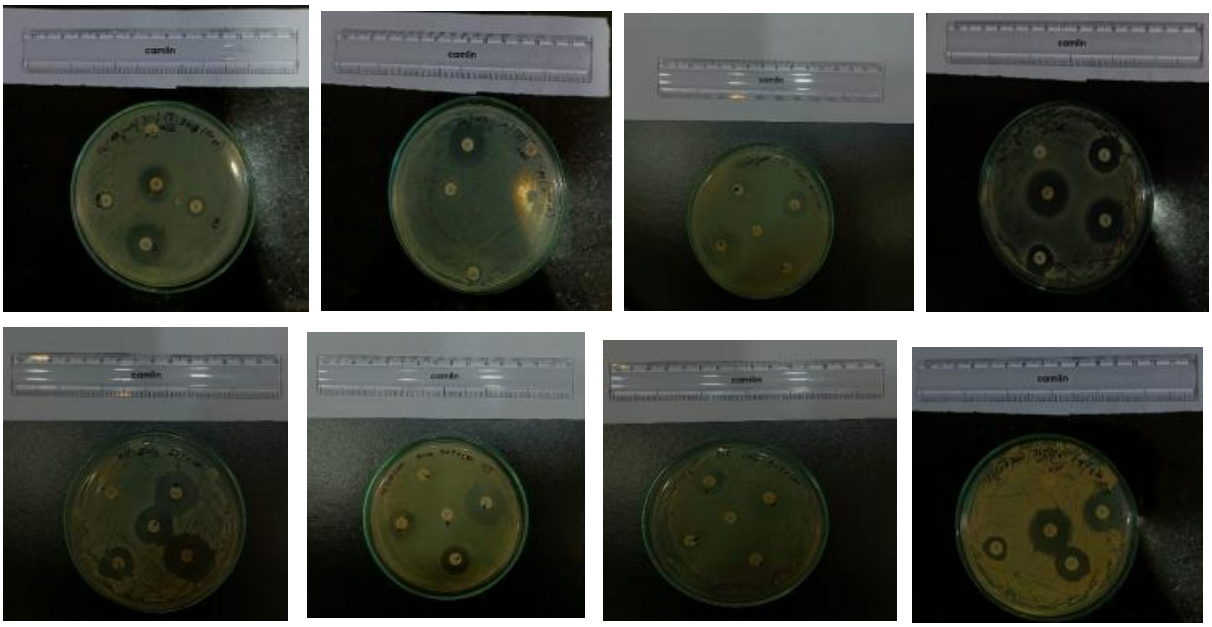

Fig57.: Kirby Bauer Disk Diffusion Susceptibility Test Results for B+T+LBL

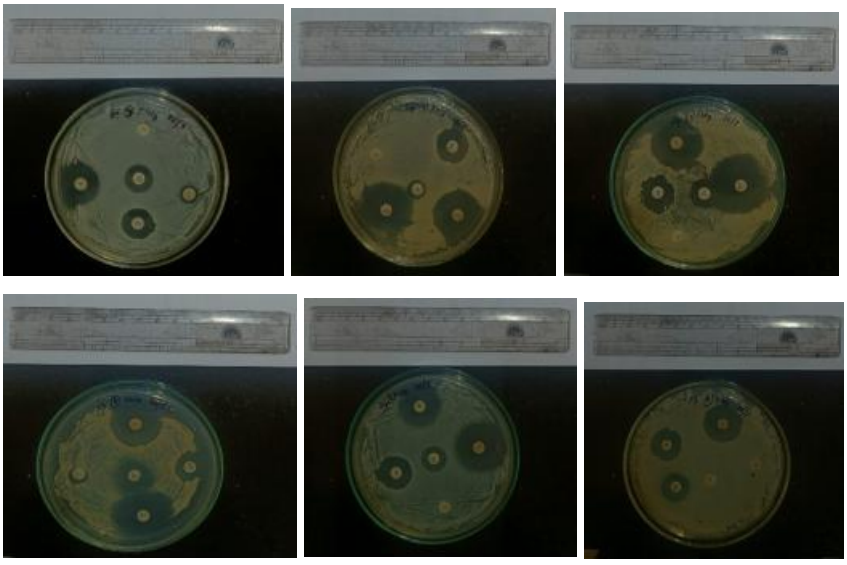

Fig58.: Kirby Bauer Disk Diffusion Susceptibility Test Results for LBL+RBL (CONTROL)

Antibiotics used: Methicillin, Ampicillin, Chloramphenicol, Vancomycin, Tetracycline

Strains tested: 17 out of 23

- Methicillin resistance: 16 out of 17 strains resistant.
- Multidrug resistance (MDR): 2 strains resistant to Methicillin, Ampicillin, Chloramphenicol, and Tetracycline
- Partial resistance: Several strains resistant to Ampicillin and Chloramphenicol
- Vancomycin: All strains were sensitive.

1. Widespread Methicillin Resistance – Suggests the presence of β-lactamase producing or methicillin-resistant organisms or altered penicillin-binding proteins. Though Methicillin is typically used to detect MRSA (Methicillin-resistant Staphylococcus aureus), catalase-negative Gram-positive cocci are unlikely to be *S. aureus. Enterococcus* spp., which was identified as likely candidates, can also show β-lactam resistance — especially *Enterococcus faecium* or resistant *E.faecalis.*
2. Multidrug Resistance (MDR) – The 2 strains resistant to four antibiotics (Methicillin, Ampicillin, Chloramphenicol, Tetracycline) could be highly resistant *Enterococcus* spp., especially nosocomial strains. Possibly resistant Gram-negative rods like *E. coli* or *Klebsiella* if these strains were TSI-positive and Gram-negative. The two multidrug-resistant strains may carry plasmid-encoded resistance genes. Could also be environmental isolates exposed to antibiotics or disinfectants
3. Ampicillin + Chloramphenicol Resistance – This combination suggests resistance mechanisms like β-lactamase production (Ampicillin resistance), Efflux pumps or enzyme inactivation (Chloramphenicol resistance). Can be resistant *Enterrococcus*.
4. Vancomycin Sensitivity – All are sensitive to Vancomycin, it rules out Vancomycin-Resistant Enterococci (VRE) — though VRE strains do exist and should be monitored if found. Confirms that Gram-positive cocci — likely *Enterococcus faecalis* or similar — are not VRE (Vancomycin-Resistant Enterococci). This is significant because VRE is a serious nosocomial pathogen, and its absence points to lesser clinical concern, or environmental/commensal strains.
5. High Methicillin Resistance, but Vancomycin Sensitivity – Indicates resistance to β-lactams, but susceptibility to glycopeptides (like Vancomycin). This profile fits with *Enterococcus faecalis* (often Methicillin/Ampicillin resistant, but Vancomycin sensitive). Also rules out MRSA, which would be catalase-positive, while the strains are catalase-negative.
6. The one strain that was sensitive to all antibiotics tested may represent – A less exposed or non-pathogenic strain or an environmental or commensal bacterium with no acquired resistance mechanisms.

Kirby-Bauer antibiotic susceptibility testing on 17 bacterial strains revealed a high prevalence of resistance to β-lactam antibiotics. Sixteen out of seventeen strains were resistant to Methicillin, and several strains also showed resistance to Ampicillin and Chloramphenicol. Two strains exhibited multidrug resistance to Methicillin, Ampicillin, Chloramphenicol, and Tetracycline. Despite this resistance, all tested strains remained sensitive to Vancomycin,

indicating the absence of Vancomycin-Resistant Enterococci (VRE). One strain displayed complete susceptibility to all five tested antibiotics, suggesting a lack of acquired resistance mechanisms. Average of zone of inhibition diameters for Methicillin, Ampicillin, Chloramphenicol, Vancomycin and Tetracyclin are 0.5cm, 0.95cm, 1.83cm, 1.08cm, 1.87cm.. The resistance profile, combined with earlier morphological and biochemical characterization, suggests that the majority of strains are likely *Enterococcus* spp. or Enterobacteriaceae such as *E. coli* or *Klebsiella* spp., with some exhibiting potential multidrug resistance mechanisms. The susceptibility to Vancomycin suggests that while these strains may pose some antibiotic resistance concerns, they remain treatable with glycopeptide-class antibiotics.

❖ *Table8.: Growing three strains on MacConkey agar*

MacConkey agar is both selective and differential, commonly used to differentiate Gram-negative bacilli enteric bacteria based on their ability to ferment lactose.

- Pink/red colonies: Lactose fermenters (acid production changes the pH, turning colonies pink)
- Colorless colonies: Non-lactose fermenters

Mouse name	No. of strains	Name of strains	Colony color
B+T+LBL	Ileum - 4	IWB	Pink
		IWS	Colorless
B+T+RFL	Colon - 4	CWB	Colorless
CONTROL	*E. coli*	*E. coli*	Pink (slow)

Fig59.: GROWING STRAINS ON MACCONKEY AGAR

Strain IWB – Pink/Red, Lactose fermenter (possibly *E. coli or Enterobacter*)

Strain IWS – Colorless, Non-lactose fermenter (e.g., *Salmonella, Proteus, Shigella*)

Strain CWB – Colorless, Non-lactose fermenter

Out of three Gram-negative bacillus strains grown on MacConkey agar, only one produced pink colonies, indicating lactose fermentation, while the remaining two produced colorless colonies, indicating they were non-lactose fermenters. *E. coli* served as a positive control and showed the expected pink colonies, but the growth was slow maybe due to lactose permease enzyme, which is encoded by lacy gene. These results suggest that the lactose-fermenting strain may be *E. coli* or a similar coliform, while the other two may belong to non-lactose fermenting genera such as *Proteus, Shigella,* or *Salmonella*.

CONCLUSION

All 23 strains formed circular, round colonies on nutrient agar. Elevation was mostly flat, some convex, margin was predominantly entire, with a few undulate. Colonies were white, yellow or orange. Most were Gram-positive cocci in clusters. Some were Gram-negative bacilli or coccobacilli.

Most strains were Indole-negative, MR-positive, VP-positive, Citrate-negative. This pattern suggests probable *Enterococcus* spp. or Enterobacteriaceae (e.g., *Enterobacter, Klebsiella*). All strains were catalase-negative indicating not Staphylococcus (rules out MRSA). Most were coagulase-negative. Most strains urease-positive suggestive of *Proteus, Klebsiella,* or *Enterococcus* (some species). TSI was performed on 14 strains. 8 strains gave positive results. 6 strains were negative. It suggests presence of fermentative enterics, likely including *E. coli, Klebsiella*, or *Enterobacter*.

In MacConkey agar, 1 strain formed pink/red colonies which means Lactose fermenter (e.g., *E. coli, Klebsiella, Enterobacter*). 2 strains formed colorless colonies indicating Non-lactose fermenters (e.g., *Salmonella, Shigella, Proteus*).

In Kirby Bauer test, Methicillin resistance was seen in 16 out of 17 strains. Ampicillin and Chloramphenicol resistance was also observed in several strains. Multidrug resistance was observed in 2 strains which was resistant to Methicillin, Ampicillin, Chloramphenicol, and Tetracycline. All strains were Vancomycin sensitive which rules out VRE (Vancomycin-Resistant Enterococci).

Bacteria isolated from the intestines of lab mice –

- Mouse LBL+RBL (anemic): Colonies recovered only from ileum
- Mouse B+T+LBL and B+T+RFL (anemic): Bacteria recovered
- Mouse LBL+RBL (healthy control): Bacteria recovered
- May suggest altered gut microbiota or bacterial depletion in anemic mouse

A total of 23 bacterial strains were isolated and characterized based on colony morphology, Gram staining, biochemical tests, and antibiotic susceptibility. All strains formed circular colonies with mostly flat elevation and entire margins, predominantly white in color. Gram staining revealed most isolates were Gram-positive cocci, while others were Gram-negative bacilli or coccobacilli. Biochemical analysis showed a common pattern of indole-negative, MR-positive, VP-positive, and citrate-negative results, with most strains being urease-positive, catalase-negative, and coagulase-negative — suggesting likely Enterococcus spp. or related

enteric bacteria. Among 14 strains tested, 8 were positive in TSI, indicating fermentative capabilities. MacConkey agar revealed one lactose-fermenting Gram-negative bacillus and two non-lactose fermenters. Antibiotic susceptibility testing showed high levels of Methicillin resistance, with a few strains also resistant to Ampicillin, Chloramphenicol, and Tetracycline, though all strains remained sensitive to Vancomycin. CWS strain of control mouse was sensitive to all antibiotics tested. Collectively, these findings reflect the presence of diverse, partially resistant enteric bacteria with potential clinical significance.

APPENDIX

Table9: Appendix A – Bacterial Recovery by Mouse Sample

Mouse name	Health status	Sample source	Bacterial recovery	Observations
LBL+RBL (ANEMIC)	Anemic	Ileum, colon	Only ileum	Colonies recovered only from ileum
B+T+LBL	Anemic	Ileum, colon	Various colonies 8	Multiple isolates observed
B+T+RFL	Anemic	Ileum, colon	Various colonies 8	Multiple isolates observed
LBL+RBL (CONTROL)	Healthy	Ileum, colon, feces	Various colonies 6	Normal bacterial growth; sensitive strain identified

Table10: Appendix B – Morphological Characteristics of Colonies

Mouse name	Name of strains	Shape	Elevation	Margin
LBL+RBL	IL, ILEUM	Circular, round	Convex	Entire
B+T+LBL	IWB	Circular, round	Flat	Undulate
B+T+LBL	IWM	Circular, round	Flat	Entire
B+T+LBL	IWS	Circular, round	Convex	Entire
B+T+LBL	IY	Circular, round	Flat	Undulate
B+T+LBL	CWB	Circular, round	Flat	Undulate
B+T+LBL	CWM	Circular, round	Flat	Entire
B+T+LBL	CWS	Circular, round	Flat	Entire
B+T+LBL	CY	Circular, round	Flat	Undulate
B+T+RFL	IWB	Circular, round	Flat	Entire
B+T+RFL	IO	Circular, round	Flat	Entire

B+T+RFL	IWS	Circular, round	Flat	Entire
B+T+RFL	IY	Circular, round	Flat	Entire
B+T+RFL	CWB	Circular, round	Flat	Entire
B+T+RFL	CO	Circular, round	Flat	Entire
B+T+RFL	CWS	Circular, round	Flat	Entire
B+T+RFL	CY	Circular, round	Flat	Entire
LBL+RBL (control)	IWB	Circular, round	Convex	Entire
LBL+RBL (control)	IWS	Circular, round	Flat	Entire
LBL+RBL (control)	CWB	Circular, round	Flat	Entire
LBL+RBL (control)	CWS	Circular, round	Flat	Entire
LBL+RBL (control)	FWB	Circular, round	Flat	Entire
LBL+RBL (control)	FWS	Circular, round	Flat	Entire

Table11: Appendix C – Summary of Biochemical Test Results

Mouse name	Strain	Indole	Methyl Red	Voges Proskauer	Citrate	Urease	Catalase	Coagulase
LBL+RBL (ANEMIC)	IL, ILEUM	_	+	_	_	_	_	_
B+T+LBL	IWB	+	_	_	_	_	_	+
B+T+LBL	IWM	+	+	+	+	_	_	+
B+T+LBL	IWS	_	+	+	_	+	_	_
B+T+LBL	IY	_	+	+	_	+	_	+
B+T+LBL	CWB	_	+	_	_	+	_	+
B+T+LBL	CWM	_	_	+	_	_	_	_
B+T+LBL	CWS	_	+	+	_	+	_	_
B+T+LBL	CY	_	_	_	_	_	_	_
B+T+RFL	IWB	_	+	_	_	_	_	_
B+T+RFL	IO	_	+	_	_	_	_	_
B+T+RFL	IWS	_	+	_	_	_	_	_
B+T+RFL	IY	_	_	_	_	_	_	_

B+T+RFL	CWB	_	_	_	_	_	_	_
B+T+RFL	CO	_	+	_	_	_	_	_
B+T+RFL	CWS	_	+	_	_	_	_	_
B+T+RFL	CY	_	_	_	_	+	_	_
LBL+RBL (CONTROL)	IWB	_	+	_	_	+	_	_
LBL+RBL (CONTROL)	IWS	_	_	_	_	+	_	+
LBL+RBL (CONTROL)	CWB	_	_	+	_	+	_	_
LBL+RBL (CONTROL)	CWS	_	_	+	_	+	_	+
LBL+RBL (CONTROL)	FWB	_	_	_	_	_	_	_
LBL+RBL (CONTROL)	FWS	_	+	_	_	+	_	+

Note: Most strains were indole-negative, MR-positive, VP-positive, citrate-negative, and urease-positive. Majority were catalase-negative and coagulase-negative.

Table12: Appendix D – Gram staining

Mouse name	Name of strains	Gram staining
LBL+RBL	IL, ILEUM	+, cocci
B+T+LBL	IWB	–, bacilli
B+T+LBL	IWM	–, bacilli
B+T+LBL	IWS	+, bacilli
B+T+LBL	IY	+, cocci
B+T+LBL	CWB	–, cocci
B+T+LBL	CWM	–, bacilli
B+T+LBL	CWS	+, cocci
B+T+LBL	CY	–, cocci
B+T+RFL	IWB	+, cocci

B+T+RFL	IO	–, cocci
B+T+RFL	IWS	–, cocci
B+T+RFL	IY	+, cocci
B+T+RFL	CWB	–, bacilli
B+T+RFL	CO	+, cocci
B+T+RFL	CWS	–, cocci
B+T+RFL	CY	+, cocci
LBL+RBL (control)	IWB	+, cocci
LBL+RBL (control)	IWS	+, cocci
LBL+RBL (control)	CWB	–, cocci
LBL+RBL (control)	CWS	+, cocci
LBL+RBL (control)	FWB	–, coccobacilli
LBL+RBL (control)	FWS	+, cocci

Table13: Appendix E – Antibiotic Susceptibility Test (Kirby-Bauer Method)

Mouse name	Strain	Methycillin	Ampicillin	Chloramphenicol	Vancomycin	Tetracycline
LBL+RBL	IL, ILEUM	R	R	R	S	R
B+T+LBL	IWB	R	S	S	S	S
B+T+LBL	IWM	R	S	S	S	R
B+T+LBL	IWS	R	R	R	S	S
B+T+LBL	IY	R	S	S	S	S
B+T+LBL	CWB	R	S	S	S	S
B+T+LBL	CWM	R	R	S	S	S
B+T+LBL	CWS	R	R	R	S	R
B+T+LBL	CY	R	S	S	S	S
LBL+RBL (control)	IWB	R	S	S	S	S
LBL+RBL (control)	IWS	R	S	S	S	S

LBL+RBL (control)	CWB	R	S	S	S	S
LBL+RBL (control)	CWS	S	S	S	S	S
LBL+RBL (control)	FWB	R	S	S	S	S
LBL+RBL (control)	FWS	R	R	S	S	S

Table14: Appendix F – MacConkey agar observations

Mouse name	Strain	Colony Color	Interpretation	Possible Identity
B+T+LBL	IWB	Pink	Lactose fermenter	*E. coli*, *Klebsiella*, *Enterobacter*
B+T+LBL	IWS	Colorless	Non-lactose fermenter	*Proteus*, *Shigella*
B+T+RFL	CWB	Colorless	Non-lactose fermenter	*Proteus*, *Shigella*

Table15: Appendix G – Summary of Total Isolates

- **Total number of isolates:** 23
- **Gram-positive cocci (clusters):** Majority
- **Gram-negative bacilli/coccobacilli:** Minority
- **Lactose fermenters on MacConkey:** 1
- **Non-lactose fermenters on MacConkey:** 2
- **Antibiotic-resistant strains:** Majority resistant to Methicillin
- **Multidrug-resistant strains:** 2
- **Vancomycin-resistant strains:** 0

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ABSTRACT

The gut microbiota is essential for maintaining host health because it aids in digestion, nutrient absorption, immunological control, and defense against pathogens. Changes in the makeup of the gut microbiota have been related to a variety of illnesses, such as hematological and metabolic problems. Low red blood cell count or hemoglobin concentration, which is the de ning feature of anemia, can affect the gut ora and, as a result, the makeup of the intestinal bacteria. The goal of this study is to compare the gut microbiome of three anemic mice with that of one healthy, non-anemic control mouse in order to nd potential variations in bacterial diversity and traits.

Bacterial extracts were taken from the gut contents of each mouse under sterile conditions in order to carry out the analysis. These extracts were then incubated for 24 hours at 37°C on nutrient agar (NA) plates using spread plating. Based on their physical features, such as size, form, color, and texture, the bacterial colonies that grew were observed and classi ed. These ndings gave early hints about the variety of microbial communities found in the gut.

The metabolic characteristics of the isolated bacteria were further determined by a variety of biochemical assays. In order to isolate Gram-negative enteric bacteria and differentiate lactose fermenters, the isolates were also grown on MacConkey agar. The Kirby-Bauer disc diffusion method was also used to assess the isolates' antibiotic susceptibility pro les. By comparing the microbial pro les of anemic and healthy mice, this study aims to identify potential alterations in the composition of the gut bacteria associated with anemia, furthering our knowledge of the relationship between gut health and disease.

KEYWORDS

Anemia, IMViC, Kirby Bauer, MacConkey, Gram staining.

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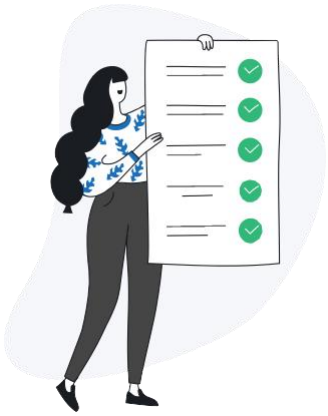

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INTRODUCTION

The gastrointestinal tract of mammals, including mice, is home to a diverse and complex microbial community that is essential to many biological functions, such as digestion, immune system regulation, and defense against harmful pathogens. The makeup and features of the gut flora of four lab mice (three anemic and one control) were examined in an experiment conducted in the current study. Three mice were taken, LBL+RBL, B+T+LBL, B+T+RFL were first made anemic by inducing it with Phenylhydrazine. A non-diseased control mouse was taken, LBL+RBL. The main goal was to isolate and identify the cultivable bacteria in the gut by extracting their intestinal contents, culturing the microorganisms on different media, and observing the resulting colonies. The use of culture-based methods, despite their limitations in identifying uncultivable species, is still necessary for identifying viable and functional bacterial groups.

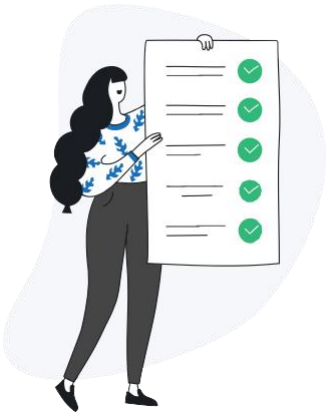
The gut bacterial contents were aseptically extracted after the mice were euthanized in a humane and sterile manner. After that, 100 microlitre of bacterial extracts were spread-plated on nutrient agar (NA) plates, which are a versatile medium that promotes the growth of a wide variety of non-fastidious species. Following a 24-hour incubation period at 37°C, which is ideal for the proliferation of many mesophilic gut bacteria, several different colonies began to grow on the agar surface. Based on characteristics including color, size, form, height, and margin, these colonies were morphologically analyzed, providing basic hints as to their identity and variety.

A battery of biochemical assays was performed in order to further identify the isolates. These tests sought to ascertain particular metabolic and enzymatic processes, which would allow for the identification of bacterial genera and species according to their physiological characteristics. Among the typical biochemical experiments were Indole test, Methyl-red test, Voges Proskauer test, Citrate utilization test, Urease test, Catalase test, Coagulase test, Triple sugar Iron (TSI), along with Gram staining was performed. Additionally, the isolates were grown on MacConkey agar, a selective and differential media intended to isolate Gram-negative rods and differentiate between lactose fermenters and non-fermenters according to the colony's color and growth patterns.

Additionally, the antibiotic susceptibility profiles of the isolated strains were evaluated using the Kirby-

Bauer disk diffusion technique. Using this method, the test organisms are incubated on agar plates that have been infected with disks impregnated with antibiotics. The susceptibility or resistance of the bacteria to particular antibiotics is indicated by the presence and size of the inhibitory zones surrounding the disks. Considering the growing antibiotic resistance, this element of the research is especially significant since gut microorganisms may act as reservoirs for resistant genes.

This study offers a fundamental knowledge of the microbial population in the mouse gut through its comprehensive methodology, which includes microbiological culturing, colony characterization, biochemical identification, selective media growth, and antibiotic susceptibility testing. The results may shed light on future research on gut health, probiotic creation, and infectious disease treatment, as well as add to the body of evidence that backs studies in microbiology, immunology, and pharmacology.



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Spread plating of 100 microlitre of bacterial extract from the gut of the three anemic mice and one control mouse was done. Several biochemical tests like Indole test, Methyl-red test, Voges Proskauer test, Citrate utilization test, Urease test, Catalase test, Coagulase test, Triple sugar Iron (TSI) test along with other tests like Kirby Bauer test, growing them on MacConkey agar and Gram staining were performed to identify the bacteria present in the gut of these four mice. All this was keeping in mind the appearance of the bacterial colonies observed with naked eye, on the Nutrient agar plates or MacConkey agar plates that they were grown.

The indole test determines if an organism can make indole, also known as benzyl pyrole, a tryptophan byproduct. Tryptophanase-producing bacteria hydrolyze tryptophan and generate ammonia, pyruvic acid, and indole. Bacteria that express the tryptophanase enzyme are able to hydrolyze and deaminate the amino acid tryptophan.

When indole interacts with the aldehyde group of para-dimethylaminobenzaldehyde (DMAB) in an acidic environment, a quinoidal red complex (rosindole) or cherry red ring is formed. This is the active ingredient in Kovác's indole detection reagent. The reagent's amyl alcohol aids in indole's trapping. Enterobacter show indole negative, while Escherichia coli show indole positive (cherry red ring). After a few seconds of applying Kovác's reagent, if a cherry red ring appears at the reagent-broth interface, it means that tryptophanase is being produced and indole is present, else if there is no cherry red ring, tryptophanase is not formed, which means indole negative result. To perform this test, a tryptophan-rich media is needed.

Methyl red is an indicator. It is used in the methyl-red test and helps to determine if a bacteria has a mixed acid fermentation pathway. Methyl red is introduced to the MRVP broth inoculation tube to serve as a pH indicator. Escherichia coli creates stable acidic end products using a mixed acid fermentation pathway. The medium's buffers will be overpowered by the acids, creating an acidic environment. The pH now hovers around 4.4. Methyl red doesn't change color when exposed to acidic conditions. As is the case with Pseudomonas sp. and Bacillus cereus, it produces orange or yellow coloring when there is less or no acid production, which is considered to be a bad outcome.

The Voges-Proskauer (VP) test is a biochemical assay utilized to detect enteric bacteria such as Escherichia, Enterobacter, and Klebsiella. This test identi es the presence of acetoin, a byproduct formed during glucose metabolism through a particular enzymatic pathway. For the test, a bacterial culture is treated with alpha-naphthol and potassium hydroxide, resulting in a red color due to the reaction with acetoin. For Klebsiella, Enterobacter, Hafnia, and Serratia, acetoin is usually the main nal product of glucose metabolism. Nonetheless, Klebsiella can display both MR+ and VP+ traits based on the surroundings. It is essential to understand that 2,3-butanediol is dif cult to detect in this assay because of its neutral characteristics, hence the emphasis is on identifying acetoin or acetyl methyl carbinol, which transforms into diacetyl with the introduction of alpha-naphthol and KOH (Barritt's reagent).

The citrate test is performed to detect bacteria that use citrate as their only carbon source. Simmone's citrate medium, utilized for this test, consists of citrate as the only carbon source. Bacteria that thrive on this medium generate an enzyme, citrate-permease, which can transform citrate into pyruvate. Pyruvate may subsequently enter the metabolic cycle of the organism to generate energy. Growth indicates the use of citrate, a key intermediate in the Krebs cycle. As bacteria uses the citrate, the ammonium salts break into ammonia, leading to a rise in alkaline environment. The change in pH changes the bromothymol blue indicator in the medium from green to blue. The only nitrogen source in the medium is Ammonium dihydrogen phosphate. Dipotassium phosphate functions as a buffer. Sodium chloride preserves the osmotic equilibrium of the solution. Sodium citrate is the only carbon source in this medium. Magnesium derived from magnesium sulfate acts as a cofactor for numerous metabolic reactions. The solidifying agent is bacteriological agar. If citrate is utilized, the microbe will gather alkaline/basic metabolites.

Urease enzyme breaks urea into ammonia and carbon dioxide. The production of urease by a microbe is utilized to identify them through biochemical testing. The pH rises as bacteria breaks the urea, which can be identi ed by the pH indicator Phenol red, which shows colour change to pink when in alkaline medium. Urease production serves as a means for Helicobacter pylori to develop opportunistic pathogenesis, utilizing a micro-alkaline environment to shield itself from the stomach's highly acidic conditions, thereby facilitating the formation of gastric ulcers. Bacteria that produce urease include Staphylococcus epidermidis. Urease production is seen by a vibrant pink (fuchsia) color on the slant.

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It is used in the methyl-red test and helps to determine if a bacteria has a mixed acid fermentation pathway.

https://study.com/academy/lesson/methyl-red-test-for-bacteria-procedure-principle.html

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The Triple Sugar Iron (TSI) test uses TSI agar as a tubed differential medium for identifying carbohydrate fermentation and H2S synthesis. In addition, gas produced by carbohydrate metabolism can be identi ed. TSI distinguishes bacteria based on their ability to ferment lactose, glucose, and sucrose, as well as on the creation of hydrogen sul de.

Glucose, sucrose, and lactose are the three sugars that make up TSI. The TSI differential medium consists of 1% lactose, 1% sucrose, 0.1% glucose, ferrous sulfate, and phenol red as a pH indicator. The capacity of test microbes to use lactose, glucose, and sucrose sugar is shown by the change in the medium's color. In an acidic environment, phenol red turns yellow, while in an alkaline environment, it turns red. To identify Gram-negative intestinal bacteria, Russell employed an agar medium containing two sugars. The third sugar sucrose is later added to this dual sugar medium to change its composition. Additionally, triple sugar medium is used to isolate enteric bacilli using ferrous sulphate which reduce sulphur, and generate carbohydrate.

The TSI test is primarily used to identify coliforms because they ferment sucrose faster than lactose. The butt of the test tube is yellow, while the slant is pink, if just glucose is fermented, which results in the production of enough acid. If the test bacteria process acidic by fermenting lactose, sucrose, or both, yellow butt and slant will be observed.

Catalase test is a quick assay. Catalase is found in the bacteria. The 3% hydrogen peroxide is applied externally. The production of catalase by the microorganism, makes hydrogen peroxide break into oxygen and water. Effervescence may be seen.

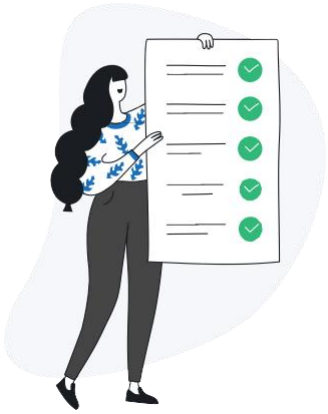
Catalase should be present in aerobic bacteria but not in anaerobic bacteria. Catalase allows one to differentiate between Gram-positive cocci - Staphylococcus aureus is catalase positive, whereas Streptococcus spp. is catalase negative. The purpose of the catalase enzyme is to counteract the antibacterial effects of hydrogen peroxide.

For coagulase test, the slide test will reveal clusters of cells. A positive reaction is indicated by clotting. Similarly, there is no correlation between the rate of coagulation and the virulence of the pathogen. A clean slide is used to conduct the slide test by creating a suspension of bacterial cells in a drop of human or rabbit plasma that has been treated with EDTA. The plasma will cause the bacterial cells to cluster if they contain bound coagulase. Due to the clumping factor being an adhesin, a member of the MSCRAMM family of adhesion molecules, the cells will bind to brinogen in the plasma, resulting in clumping. This will cause the bacterial cells to clump together noticeably on the microscope slide.

The Gram stain is essential for determining the phenotypic characteristics of bacteria. According to cell wall structure, the staining technique distinguishes species in the domain Bacteria. Due to their dense peptidoglycan layer, gram-positive cells appear purple after being stained. Since Gram-negative cells have a thin peptidoglycan layer, they stain reddish orangish.

Crystal violet dissociates into CV+ and Cl- ions in aqueous solutions, which can pass through the cell walls and membranes of both type of cells. When iodine (I- or I3 -) is introduced, it interacts with CV+

to create big CVI complexes inside the cytoplasm and outside layers of the cell. The CV+ stains the cells purple by interacting with negatively charged elements of bacterial cells. The decolorizing agent, which is either ethanol or a combination of ethanol and acetone, interacts with the lipids in the membranes of both Gram-positive and Gram-negative bacteria. The gram-negative cell's outer membrane comes off, leaving the peptidoglycan layer exposed. Ethanol makes Gram-negative cell walls permeable, so the big CV-I complexes goes out of the cell. The addition of ethanol dehydrates the gram-positive cell's heavily cross-linked and multilayered peptidoglycan. The multi-layered aspect of the peptidoglycan, along with the dehydration caused by the ethanol treatment, traps the large CV-I complexes inside the cell. The gram-positive cell retains its purple color following decolorization, but the gram-negative cell loses it and is only seen when the positively charged dye safranin is introduced as a counterstain.



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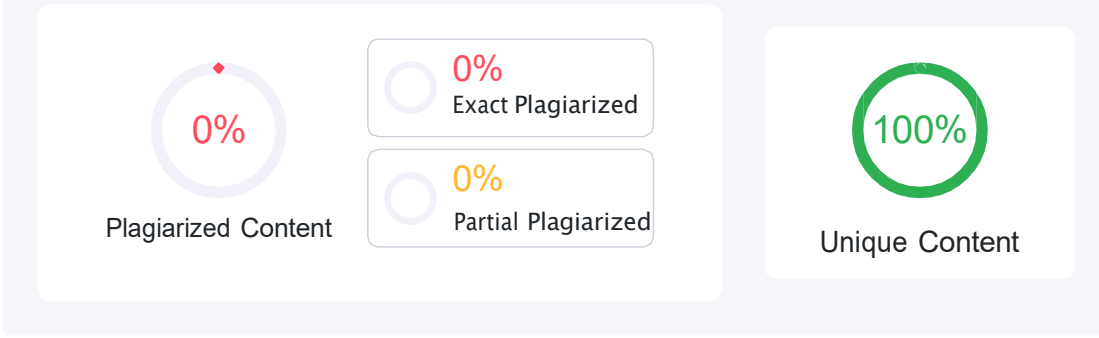

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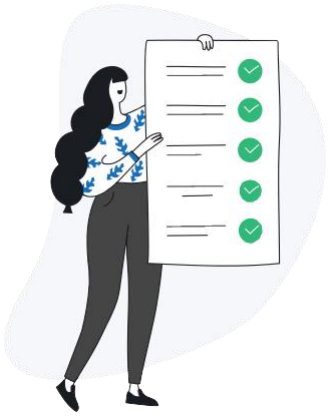
The Kirby-Bauer technique or disc diffusion is an antibiotic susceptibility test that helps decide which antibiotic to use while treating an infection. In this method, Iter paper circles with a 6mm diameter are saturated with known amounts of various antimicrobial chemicals. The test organism is introduced onto Nutrient Agar (NA) plates, and these disks are positioned on top of the plates. The antimicrobial molecule will diffuse at the same time that the test organism grows. The capacity of that antimicrobial molecule to prevent the growth of the test organism is determined by whether or not the organism grows around the discs.

The following variables in uence how quickly the antibacterial substance diffuses:

- The molecular weight of the antimicrobial substance. The size of the molecules is inversely proportional to their diffusion rate on the agar.
- The drug's solubility and diffusion characteristics in agar media.

Gram-negative rods belonging to the Enterobacteriaceae family, can be grown on MacConkey and hence can be distinguished where MacConkey agar acts as a differential and selective medium. It contains bile salts and crystal violet, which stop the growth of gram-positive bacteria and promoting the growth of gram-negative bacilli. Lactose fermenters that produce reddish pinkish colonies and non-fermenters that produce colorless or transparent colonies can be distinguished using MacConkey agar.

From the gut samples of four mice, a total of 23 different bacterial species were identi ed. Among the mice and the sampled gut regions (ileum, colon, and feces where applicable), the distribution of these strains varied greatly. From the ileum region of the rst mouse (LBL+RBL), only one strain was discovered, and none were discovered in the colon. With four strains identi ed from the ileum and another four from the colon, the second mouse (B+T+LBL) had greater microbial diversity. The third mouse (B+T+RFL) likewise displayed four strains from each of the ileum and colon, demonstrating a well-balanced and diverse microbial community in both locations. The fourth mouse (control, LBL+RBL) exhibited moderate diversity, with two strains isolated from the ileum, two from the colon, and two additional strains from its feces. The signi cant variation in gut bacterial makeup between the individual mice revealed by this comparative distribution may be related to physiological variables, such as disease state, notably anemia in three of the four mice. The rst mouse's extraordinarily low microbial count might point to a disrupted or repressed gut microbiota, which could be related to its anemia.



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INDOLE TEST
Materials –
The primary criterion for a good indole test medium is that it has enough tryptophan. Tryptone broth is frequently utilized, albeit many media ful l this requirement.
Making Tryptophan broth is a must.
10.0 g of tryptone
5.0 g of sodium chloride
The ingredients should be dissolved in 1000 millilitre of sterile water at pH 7.4. Give each tube 4 ml. Autoclave the tubes for 15 minutes at 121°C under 15 psi pressure after capping them. Keep the tubes in the fridge at 4 to 10°C.
Kovács reagent preparation
150.0 mL of reagent grade amyl or isoamyl alcohol (butyl alcohol may be used instead)
10.0 g of para-dimethylaminobenzaldehyde (DMAB)
50.0 mL of concentrated HCl
To dissolve DMAB in the alcohol, you may need to apply a little heat to help the aldehyde dissolve. Gradually introduce the acid to the aldehyde-alcohol mixture. The remedy is only stable for a little period and should be a light yellow hue. The market also sells Kovács reagent.
There were 23 strains in total, plus one positive control E.coli.
Making of 75 mL Tryptophan broth.
0.75 g of tryptone used
0.375 g of NaCl used
75 mL distilled water
For this experiment, the Kovács reagent from the market was used.
Methods –
Each test tubes had 3 mL of tryptophan broth.
Little bit of a pure culture was introduced using an inoculating loop into the tryptone broth test tube.
Incubation temperature between 33°C and 37°C for 24 to 48 hours was maintained.
5 drops of Kovács reagent was added directly to the tube to check for indole synthesis.
The formation of cherry-red ring in the reagent layer above the media suggests a positive indole test.
The reagent layer will be yellow or somewhat hazy if a culture is indole negative.

METHYL RED TEST
Materials –
Inoculation loop
Methyl red (MR)
Making Buffered Glucose Broth for 1000 mL dH2O

Peptone – 10 g
NaCl – 5 grams
Disodium hydrogen phosphate – 3.5 g
Potassium dihydrogen phosphate – 1.5 g
pH 7.2
Methyl red indicator: 0.1 MR dissolved in 200 ml of 95% ethanol.
There were 23 strains for MR test in total, plus one positive control E.coli, for MR and one positive control for VP test.
Making of 130 mL Buffered Glucose broth (preparing for both MR and VP test).
Peptone – 1.3 g
NaCl – 0.65 grams
Disodium hydrogen phosphate – 0.455 g
Potassium dihydrogen phosphate – 0.195 g
Distilled water – 130 mL

Methods –
1. Buffered Glucose Broth is prepared and autoclaved.
2. The test tubes are filled with 2.5 mL autoclaved media.
3. In separate test tubes containing media, each organism is inoculated.
4. The test tubes are correctly labeled and kept at 37°C for 24–48 hours.
5. Methyl red 5 to 6 drops are put into the medium, and the results noted.

VOGES PROSKAUER TEST
Materials –
Making Buffered Glucose Broth for 1000 mL dH2O
Peptone – 10 g
NaCl – 5 grams
Disodium hydrogen phosphate – 3.5 g
Potassium dihydrogen phosphate – 1.5 g
pH 7.2

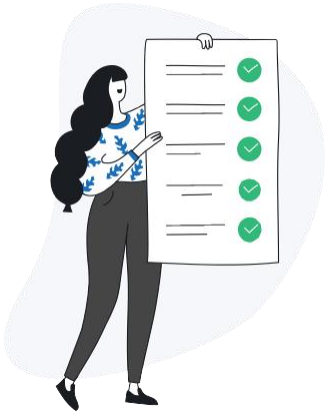
Reagents
VP Reagent A: Barritt's Reagent A
Alpha Naphthol (5%): 5g
Absolute Ethanol: 1000ml
VP Reagent B: Barritt's Reagent B
KOH:400g
Deionized Water: 1000ml
Making of 130 mL Buffered Glucose broth (preparing for both MR and VP test).
Peptone – 1.3 g
NaCl – 0.65 grams
Disodium hydrogen phosphate – 0.455 g
Potassium dihydrogen phosphate – 0.195 g
Distilled water – 130 mL
Methods –
1. Around 2.5-3 mL media is poured into test tubes.
2. Using a sterile inoculation loop, the different organisms are inoculated into the test tubes containing the media.
3. It is kept at 37°C for 24–48 hours.
4. The next day, the test tube is gently shaken after the addition of just a few drops of the two reagents: 6 drops of Barritt's reagent A, which is 5% α-napthol in absolute alcohol, and 2 drops of Barritt's reagent B, which is 40% KOH.

CITRATE UTILIZATION TEST
Materials –
Simmons Citrate Agar:
Sodium Chloride (NaCl) – 5.0g
Sodium Citrate (dehydrate) – 2.0g
Ammonium Dihydrogen Phosphate – 1.0g

Dipotassium Phosphate – 1.0g
Magnesium Sulfate (heptahydrate) – 0.2g
Bromothymol Blue – 0.08g
Agar – 15g
Deionized water = 1000 ml
Final pH 6.9 +/- 0.2 at 25°C.
There are 23 strains in total, plus one Citrate positive control.
Making 130 mL Simmons Citrate Agar:
Sodium Chloride (NaCl) – 0.65g
Sodium Citrate (dehydrate) – 0.26g
Ammonium Dihydrogen Phosphate – 0.13g
Dipotassium Phosphate – 0.13g
Magnesium Sulfate (heptahydrate) – 0.026g
Bromothymol Blue – 0.0104g
Agar – 1.95g
Distilled water – 130mL

Methods –
In distilled water, the salts are dissolved.
Bromothymol blue and agar are introduced.

3. Till the agar melted, it was gently heated while stirring.
4. Petri dishes can use the medium as a plate medium, whereas test tubes can utilize it as slopes. In both cases, the medium's surface is softly inoculated by streaking.
5. Deep green media is dispensed into 16-mm tubes at a rate of 4.0 to 5.0 ml per tube.
6. Autoclaving is performed for 15 minutes at 121°C under 15 psi pressure.
7. The tubes are cooled at an angle (with a lengthy slope and a low butt).



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UREASE TEST
Materials –
Christensen Urea Agar for 1000 mL distilled water
• Peptone – 1 g
• Dextrose – 1 g
• Sodium chloride – 5 g
• Potassium phosphate, monobasic – 2 g
• Urea – 20 g
• Phenol red – 0.012 g
• Agar – 15 to 20 g
The rst six components were dissolved in 100 mL of distilled water. Autoclaving of the agar after it has been boiled and thoroughly dissolved in 900 ml of distilled water at 121°C and 15 psi for 15 minutes, was done. In a sterile manner, 100 ml of lter-sterilized urea base was added to the chilled agar solution, mixing well. 5 ml of media poured into each sterile tube (13 x 100 mm) and tilt the tubes throughout cooling until they are rm. Prepared media was refrigerated at 4 to 8°C. No reheating of the medium after it has been prepared since the urea will break down if reheated. There are 23 strains in total, plus one Urease Enterobacter positive control.
Christensen Urea Agar for 130 mL
• Peptone – 0.13 g
• Dextrose – 0.13 g
• Sodium chloride – 0.65 g
• Potassium phosphate, monobasic – 0.26 g
• Urea – 2.6 g
• Phenol red – 0.00156 g
• Agar – 1.95g
• Distilled water – 130mL
So, rst six components were dissolved in 13 mL of distilled water. Autoclaving of the agar after it has been boiled and thoroughly dissolved in 117 ml of distilled water at 121°C and 15 psi for 15 minutes, was done. In a sterile manner, 13 ml of lter-sterilized urea base was added to the chilled agar solution, mixing well. 5 ml of media poured into each sterile tube (13 x 100 mm) and tilt the tubes throughout cooling until they are rm.
Methods –
1. The entire slant surface was streaked, from pure culture.
2. Place tubes with loose lids in a 35°C incubator.
3. Examine the slope for a shift in color at 6 hours, 24 hours, and every day for a period of six days.

TRIPLE SUGAR IRON (TSI) TEST

Materials –

TSI agar preparation

- Beef extract – 3.0g
- Yeast extract – 3.0g
- Peptone – 20g
- Glucose – 1.0 g
- Lactose – 10.0 g
- Sucrose – 10.0 g
- Ferrous sulphate – 0.2 g
- NaCl – 5 g
- Sodium thiosulfate – 0.3 g
- Phenol red – 0.024 g
- Agar – 13.0 g
- Distilled water – 1000 mL

Combine ingredients, and adjust the pH to 7.3. Autoclaving at 121°C for 15 min. Cooling in a slanted position.

There were 14 strains in total, with one E.coli positive control.

100 mL TSI agar preparation

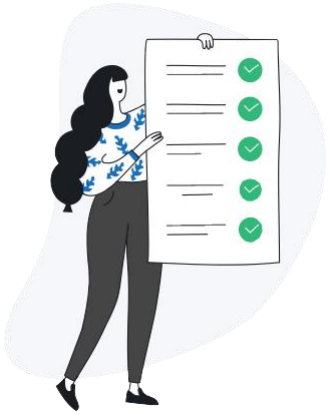
- Beef extract – 0.3g
- Yeast extract – 0.3g
- Peptone – 2.0g
- Glucose – 0.1 g
- Lactose – 1.0 g
- Sucrose – 1.0 g
- Ferrous sulfate – 0.02 g
- NaCl – 0.5 g
- Sodium thiosulfate – 0.03 g
- Phenol red – 0.0024 g
- Agar – 1.3 g
- Distilled water – 100 mL

Methods –

1. An isolated colony picked up using a straight inoculating needle.
2. To inoculate the TSI slant, sticking the butt of the needle down to the bottom done, then the needle was pulled back, and then the surface of the slant was streaked.
3. Loose closing to allow air access was done. Incubation at 37°C for 24 hours. Then results were read.

The reactions can produce three different kinds of data.

- Sugar fermentations: yellow acidic butt, red basic slant, glucose fermented but sucrose or lactose haven't. Lactose and/or sucrose fermentation, yellow acidic butt, and yellow acidic slant. Red alk. if no fermentation of glucose, lactose, or sucrose.
- Gas production, which is evidenced by bubbles in the buttocks. The agar could break or be pushed up by a lot of gas.
- Hydrogen sul de generation - The formation of black ferrous sul de from the interaction of H2S with the ferrous ammonium sulfate causes the butt to turn black, indicating that hydrogen sul de is produced from thiosulfate. The bacteria's capacity to create hydrogen sul de (H2S) from sodium thiosulfate is shown by the black precipitate. Since H2S is colorless, ferric ammonium citrate is employed as an indicator, which causes the production of insoluble ferrous sul de. An acidic environment is necessary for the creation of H2S, and although the black precipitate makes it impossible to see a yellow butt, the butt is still acidic. Acid over acid (A/A) and H2S positive are the ways the outcomes will be documented.



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CATALASE TEST

Materials –

3% hydrogen peroxide is used, which is readily available for purchase, for regular aerobic testing. Hydrogen peroxide is kept in a dark bottle in the refrigerator. A 15% H2O2 solution is required to identify anaerobic bacteria. For 23 strains and one positive S.aureus, the test is performed.

Methods –

1. A clean glass slide is divided into several sections, one for the experiment and one for the control.
2. A tiny drop of regular saline solution was put on each area. A little amount of bacterial culture with a sterile inoculating loop was taken and spread over the saline drop to form a uniform suspension.
3. Using a Pasteur pipette, one drop of 3% hydrogen peroxide was put to the test smear.
4. The slide was checked right away for the development of gas bubbles.

COAGULASE TEST

Materials –

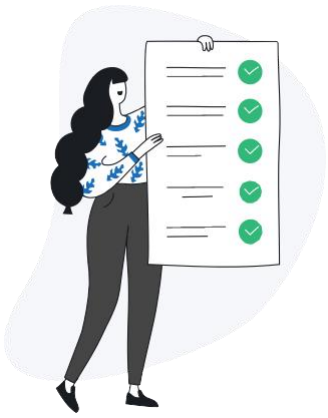
As lyophilized citrated or EDTA-treated rabbit plasma, commercially available coagulase plasma is purchased from a provider and rehydrated with sterile distilled water just before usage. As previously stated, EDTA therapy is preferable. The manufacturer will specify the quantity of water required for rehydration. Since plasma is inactivated by cycles of thawing and refreezing, frozen aliquots should be kept in a freezer that is not frost-free.

- Overnight plate culture of the organism to be tested on noninhibitory agar, utilizing a microscope slide
- Control organisms, typically comprised S. aureus (positive control).
- Physiological saline or water prepared with 0.09 g of NaCl in 10 mL of distilled water, and autoclaved.
- Unsterilized dropper
- Reconstituted plasma coagulase
- Sterile pipette

Methods –

1. A tiny drop (10-20 microlitre) of water or saline was put into the slide using the nonsterile dropper. The water or saline serves as a control to make sure that the strain readily emulsi es rather than auto-agglutinating and clumping with itself.
2. A tiny drop of reconstituted plasma was put into the circle at the other end of the slide using the sterile dropper.
3. Using the loop or applicator stick, cells from one colony were taken and emulsi ed in the water (or saline) and then in the drop of plasma. The sequence is crucial to prevent trace quantities of plasma

from being transferred into the control drop.
4. Clumping within 10 seconds of introducing the bacterial cells to the plasma is an indicator of coagulase positive organism. If the slide is gently rocked in a gure 8 manner, the clumping will become more apparent.



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GRAM STAINING

Materials –

Crystal Violet
Methylene Blue
Potassium Iodide
Ethanol, 95%
Acetone
Ammonium oxalate
Iodine
Sodium bicarbonate
Safranin O
Bunsen Burner
Microscope
Slide
Distilled water

Methods –

Making a slide smear.

1. To take a culture from a petri dish, a tiny amount of a colony from the petri dish was aseptically transfered onto the slide after adding a drop of water.
2. Using an inoculation loop, the culture is distributed to form an uniform, thin layer over a circle that is 1.5 cm in diameter (about the size of a dime).
3. A sterile forcep was used to hold the slide.

Gram staining

1. Few drops of cv dye was put, and allowed to stand for 60 seconds.
2. With a stream of distilled water, the stain was washed away and any remaining stain. The aim of this procedure is to get rid of the stain, not the set culture.
3. Then iodine solution was put on the smear, using around 5 drops, or enough to coat the xed culture, and allowed to sit for sixty seconds.
4. Washing of the the slides under running water was done, after pouring out the iodine solution to get rid of any extra water on the surface.
5. Few drops of alcohol acetone decolorizer was put to the solution so that it drips down the slide. After thirty seconds, it was rinsed with water. When the solvent is no longer colored as it runs over the slide, washing was stopped.
6. After 20 seconds, counterstaining with 5 drops of the Safranin solution was done.
7. Rinsing of the red Safranin solution was done with water. Bibulous paper was used to absorb extra

water.

8. Immediate rinsing off any spilled stain with lots of water was done, to prevent lasting stains on the sink, lab bench, or glassware.

9. Observation of the completed slide under a microscope was done.

KIRBY BAUER DISK DIFFUSION SUSCEPTIBILITY TEST

Materials –

Nutrient Agar – 28.0 g in 1000 mL distilled water

Nutrient broth – 13.0 g in 1000 mL distilled water

Petri plates

Antibiotic Discs (In our experiment we used Penicillin, Tetracycline, Streptomycin and Chloramphenicol)

Forceps

Inoculum

Metric ruler or Calipers

Preparation of Nutrient agar plates

- Nutrient agar required for 300 mL of distilled water – 8.4 g
- After autoclaving at 15psi for 15 minutes at 121℃, the nutrient agar was poured in the petri dishes.
- After it solidi ed, it was put in the incubator overnight, at 37℃.

Preparation of Nutrient agar broth

- NB for 50 mL of dH2O – 0.65 g. autoclave.

Methods –

1. The organisms must be in the log phase for the data to be accurate. In order to ensure that the test organism's subcultures are maintained in nutrient broth medium, they must be created the day before. Once the O.D of the culture measured at 0.5, the culture can be spread on the nutrient agar plates.
2. Making the NA plates: The plates should have a thickness of 4 mm (roughly 20-25 ml of liquid agar for 100-mm plates). The agar medium should be exactly 4 mm thick since the zone of inhibition will be smaller the thicker it is. The pH of the agar should be between 7.2 and 7.4 at ambient temperature.
3. Using a spreader, 100 microlitre of bacterial culture was introduced onto the agar plates and evenly distributed. Then I let it wait for 5-10 minutes.
4. The antibiotic discs were placed on the agar with a forcep.
5. The plates are positioned so that their zone of inhibition does not overlap.
6. After positioning all of the discs, the plates are incubated for 16 to 18 hours at 37°C.

MACCONKEY AGAR PLATES

Materials –

MacConkey Agar – 51.53 g in 1000 mL distilled water

Petri plates

Preparation of MacConkey agar plate

- MacConkey Agar for 20 mL dH2O – 1.0306 g
- After autoclaving at 15psi for 15 minutes at 121℃, the media was poured in the petri dish.
- After it solidi ed, it was put in the incubator overnight, at 37℃.

Methods –

1. A plate was divided into four segments.
2. The Gram-negative rods, along with a control (E.coli), were streaked on the MacConkey agar plate, using sterile inoculating loop.
3. The plate was kept in the incubator for 24-48 hours, at 37℃.

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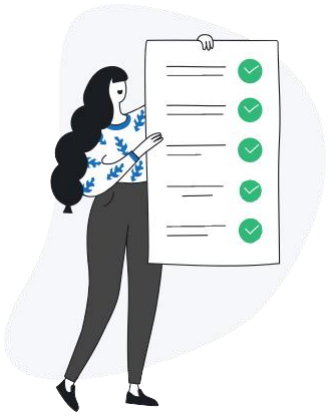

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The colony morphology of the 23 bacterial strains isolated and cultured on nutrient agar plates revealed consistent and notable characteristics. All observed colonies exhibited a circular, round shape, indicating a uniform growth pattern across the different strains. The colony margins were predominantly entire, with some strains showing undulate edges. Color-wise, most of the bacterial colonies were white, while a smaller number displayed yellow or orange pigmentation. These morphological features–shape, elevation, margin–provide preliminary insight into the diversity and identity of the bacterial strains and serve as an important basis for further microbiological characterization.

Interestingly, no bacterial colonies were observed from the colon sample of Mouse LBL+RBL, which was an anemic mouse. This absence of growth may be associated with altered gut microbiota or reduced bacterial load due to the disease condition. The rst three mice, LBL+RBL, B+T+LBL, B+T+RFL, were anemic, while Mouse LBL+RBL (CONTROL) served as a non-disease control. This observation may point to a possible impact of anemia on the composition or viability of gut microbiota.

In standard Enterobacteriaceae differentiation: MR-positive indicates mixed acid fermentation, seen in E. coli, Shigella, and others. Rarely are both MR and VP strongly positive in the same strain.

So if most of the isolates are showing both MR and VP positivity, this suggests one or more of the following:

• Mixed fermentation capabilities, possibly pointing toward intermediate fermenters or environmental enteric bacteria with variable fermentation pathways.

• Non-Enterobacteriaceae strains that may exhibit both MR and VP positivity due to unique metabolic traits.

• Slight cross-reactivity or borderline results–sometimes, weak VP or MR reactions may give a false sense of dual-positivity, depending on incubation and interpretation timing.

Most strains show citrate negative. This indicates that most of the strains cannot utilize citrate as the sole carbon source, ruling out typical Enterobacter or Klebsiella, which are usually citrate positive. The bacterial strains isolated from mice gut microbiota may predominantly consist of non-pathogenic or commensal bacteria that: Carry out mixed acid fermentation (MR+), have limited ability to use citrate (Citrate−), show some acetoin production (VP+), do not produce indole (Indole−), likely due to lack of tryptophanase enzyme.

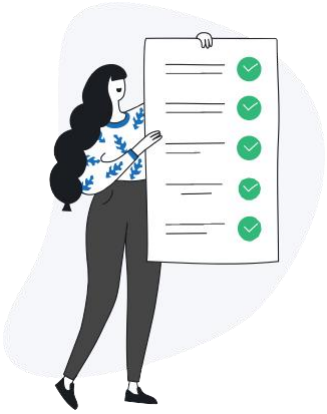
This is unusual because most organisms do not show both MR and VP positive. That typically suggests metabolic exibility–i.e., the bacteria can carry out both mixed acid and butylene glycol fermentation to some degree.

Possible bacteria can be environmental Escherichia coli as some E. coli strains, especially commensal gut isolates, may show weak VP positivity under certain conditions. They are often MR+, Citrate−, and some may be Indole−. It can also be a less common Enterobacteriaceae or closely related genera like

Hafnia or Pantoea may exhibit mixed IMViC results and are found in gut ora or the environment. Most strains are urease positive. Urease hydrolyzes urea into ammonia and CO_2. Most strains show coagulase negative. All Strains are catalase negative. Catalase-negative bacteria are typically Streptococci, Enterococci, and some anaerobes. Since the strains are catalase negative, these are not Staphylococci, so this result further con rms that.
The most striking and narrowing trait here is Catalase Negative in all strains, which signi cantly narrows it down. When combined with: Urease Positive, Coagulase Negative, it can most likely be a Gram-positive cocci, speci cally in the genera:

Enterococcus spp. – Catalase negative, some strains are urease positive, non-hemolytic or γ-hemolytic usually, found in gut ora, especially in humans and animals, often coagulase negative, associated with both health and opportunistic infections.
Streptococcus spp. – Catalase negative, typically urease negative, but some environmental or commensal species may show variable urease activity.
Anaerobic cocci (e.g., Peptostreptococcus) – Catalase negative, may be urease positive, commensals of the gut and mouth.

The most likely candidates are Enterococcus species, possibly Enterococcus faecalis or Enterococcus durans, which are common gut commensals and t this biochemical pro le. These are often facultative anaerobes, survive well in nutrient agar, and are known to show urease activity in some strains.
Triple Sugar Iron (TSI) test is a powerful tool to evaluate carbohydrate fermentation (glucose, lactose, sucrose) and hydrogen sul de (H_2S) production in Gram-negative enteric bacteria. 14 out of 23 strains were tested. 8 are positive, could be Escherichia coli, Enterobacter spp.
A positive TSI test typically refers to: Acid production (seen as yellow color changes), gas production (bubbles/cracks in the agar), hydrogen sul de (H_2S) production (black precipitate). So, a positive result usually means that the bacteria can: Ferment glucose (and possibly lactose/sucrose), possibly produce gas or H_2S, likely belong to Enterobacteriaceae or related fermentative Gram-negative rods.
A negative TSI test means: No sugar fermentation (no color change or slant/butt stays red), no acid/gas/H_2S production. This suggests that the bacteria do not ferment glucose, lactose, or sucrose, may be non-enteric, could be Gram-positive cocci like Streptococcus or Enterococcus (they often don't grow well or react clearly on TSI slants), or non-fermenting Gram-negative rods like Pseudomonas.
These 6 strains could be: Enterococcus spp., Streptococcus spp.
The 8 TSI-positive strains likely include fermentative Gram-negative enteric bacteria, most plausibly from the Enterobacteriaceae family (E. coli, Klebsiella, etc.).
The 6 TSI-negative strains may represent Gram-positive cocci like Enterococcus spp. or non-fermenting bacteria that do not utilize the sugars in the TSI medium.



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Most strains: Gram-positive cocci in clusters or tetrads
Some strains: Gram-negative cocci in clusters
1 strain: Gram-negative cocci in pairs
4 strains: Gram-negative bacilli

1. Gram-Positive Cocci in Clusters: These are likely staphylococci or similar genera. But you noted all were catalase-negative, which rules out Staphylococcus. Possibilities include: Enterococcus spp. (though often diplococci or chains, but variable). Since they're urease positive and catalase negative, they are most likely Enterococcus spp.
2. Gram-Negative Cocci in Clusters: Gram-negative cocci are uncommon; when present in clusters, possibilities include: Moraxella spp. However, clusters are not typical for these genera – this may be a staining or arrangement artifact. If catalase-negative: possibly misidenti ed coccoid rods, or fastidious bacteria. These could also be fragile Gram-negative rods that appeared coccoid–seen with Haemophilus or stressed cells.
3. Gram-Negative Bacilli: These could be Enterobacteriaceae, like E. coli, Klebsiella, Enterobacter, etc. TSI-positive strains probably fall into this group.

These are non-classical enteric Gram-negatives, possibly stressed or slow-growing. That's why they may be catalase negative.
Colony morphology, TSI, IMViC, and urease tests collectively point toward:
Enterococcus (Gram+ cocci), Enterobacteriaceae (Gram− rods, from TSI+ strains)
Antibiotics used: Methicillin, Ampicillin, Chloramphenicol, Vancomycin, Tetracycline
Strains tested: 17 out of 23

- Methicillin resistance: 16 out of 17 strains resistant
- Multidrug resistance (MDR): 2 strains resistant to Methicillin, Ampicillin, Chloramphenicol, and Tetracycline
- Partial resistance: Several strains resistant to Ampicillin and Chloramphenicol
- Vancomycin: All strains were sensitive.

1. Widespread Methicillin Resistance - Suggests the presence of β-lactamase producing or methicillin-resistant organisms or altered penicillin-binding proteins. Though Methicillin is typically used to detect MRSA (Methicillin-resistant Staphylococcus aureus), catalase-negative Gram-positive cocci are unlikely to be S. aureus. Enterococcus spp., which was identi ed as likely candidates, can also show β-lactam resistance – especially Enterococcus faecium or resistant E.faecalis.
2. Multidrug Resistance (MDR) - The 2 strains resistant to four antibiotics (Methicillin, Ampicillin, Chloramphenicol, Tetracycline) could be highly resistant Enterococcus spp., especially nosocomial strains. Possibly resistant Gram-negative rods like E. coli or Klebsiella if these strains were TSI-positive and Gram-negative. The two multidrug-resistant strains may carry plasmid-encoded

resistance genes. Could also be environmental isolates exposed to antibiotics or disinfectants

3. Ampicillin + Chloramphenicol Resistance – This combination suggests resistance mechanisms like β-lactamase production (Ampicillin resistance), Ef ux pumps or enzyme inactivation (Chloramphenicol resistance). Can be resistant Enterrococcus.

4. Vancomycin Sensitivity – All are sensitive to Vancomycin, it rules out Vancomycin-Resistant Enterococci (VRE) — though VRE strains do exist and should be monitored if found. Con rms that Gram-positive cocci – likely Enterococcus faecalis or similar – are not VRE (Vancomycin-Resistant Enterococci). This is signi cant because VRE is a serious nosocomial pathogen, and its absence points to lesser clinical concern, or environmental/commensal strains.

5. High Methicillin Resistance, but Vancomycin Sensitivity – Indicates resistance to β-lactams, but susceptibility to glycopeptides (like Vancomycin). This pro le ts with Enterococcus faecalis (often Methicillin/Ampicillin resistant, but Vancomycin sensitive). Also rules out MRSA, which would be catalase-positive, while the strains are catalase-negative.

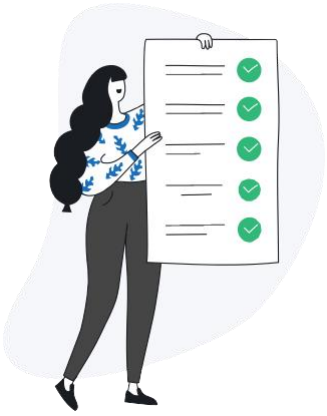
6. The one strain that was sensitive to all antibiotics tested may represent – A less exposed or non-pathogenic strain or an environmental or commensal bacterium with no acquired resistance mechanisms.

Kirby-Bauer antibiotic susceptibility testing on 17 bacterial strains revealed a high prevalence of resistance to β-lactam antibiotics. Sixteen out of seventeen strains were resistant to Methicillin, and several strains also showed resistance to Ampicillin and Chloramphenicol. Two strains exhibited multidrug resistance to Methicillin, Ampicillin, Chloramphenicol, and Tetracycline. Despite this resistance, all tested strains remained sensitive to Vancomycin, indicating the absence of Vancomycin-Resistant Enterococci (VRE). One strain displayed complete susceptibility to all ve tested antibiotics, suggesting a lack of acquired resistance mechanisms.

The resistance pro le, combined with earlier morphological and biochemical characterization, suggests that the majority of strains are likely Enterococcus spp. or Enterobacteriaceae such as E. coli or Klebsiella spp., with some exhibiting potential multidrug resistance mechanisms. The susceptibility to Vancomycin suggests that while these strains may pose some antibiotic resistance concerns, they remain treatable with glycopeptide-class antibiotics.

Strain IWB – Pink/Red, Lactose fermenter (possibly E. coli or Enterobacter)
Strain IWS – Colorless, Non-lactose fermenter (e.g., Salmonella, Proteus, Shigella)
Strain CWB – Colorless, Non-lactose fermenter

Out of three Gram-negative bacillus strains grown on MacConkey agar, only one produced pink colonies, indicating lactose fermentation, while the remaining two produced colorless colonies, indicating they were non-lactose fermenters. E. coli served as a positive control and showed the expected pink colonies, but the growth was slow maybe due to lactose permease enzyme, which is encoded by lacy gene. These results suggest that the lactose-fermenting strain may be E. coli or a similar coliform, while the other two may belong to non-lactose fermenting genera such as Proteus, Shigella, or Salmonella.



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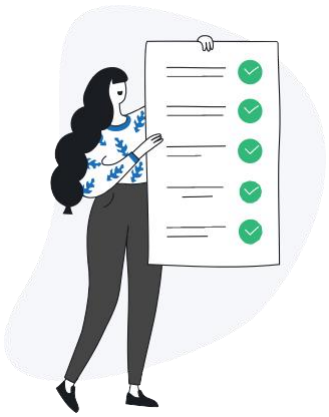
All 23 strains formed circular, round colonies on nutrient agar. Elevation was mostly at, some convex, margin was predominantly entire, with a few undulate. Colonies were white, yellow or orange. Most were Gram-positive cocci in clusters. Some were Gram-negative bacilli or coccobacilli.
Most strains were Indole-negative, MR-positive, VP-positive, Citrate-negative. This pattern suggests probable Enterococcus spp. or Enterobacteriaceae (e.g., Enterobacter, Klebsiella). All strains were catalase-negative indicating not Staphylococcus (rules out MRSA). Most were coagulase-negative. Most strains urease-positive suggestive of Proteus, Klebsiella, or Enterococcus (some species). TSI was performed on 14 strains. 8 strains gave positive results. 6 strains were negative. It suggests presence of fermentative enterics, likely including E. coli, Klebsiella, or Enterobacter.
In MacConkey agar, 1 strain formed pink/red colonies which means Lactose fermenter (e.g., E. coli, Klebsiella, Enterobacter). 2 strains formed colorless colonies indicating Non-lactose fermenters (e.g., Salmonella, Shigella, Proteus).
In Kirby Bauer test, Methicillin resistance was seen in 16 out of 17 strains. Ampicillin and Chloramphenicol resistance was also observed in several strains. Multidrug resistance was observed in 2 strains which was resistant to Methicillin, Ampicillin, Chloramphenicol, and Tetracycline. All strains were Vancomycin sensitive which rules out VRE (Vancomycin-Resistant Enterococci).
Bacteria isolated from the intestines of lab mice –
• Mouse LBL+RBL (anemic): Colonies recovered only from ileum
• Mouse B+T+LBL and B+T+RFL (anemic): Bacteria recovered
• Mouse LBL+RBL (healthy control): Bacteria recovered
• May suggest altered gut microbiota or bacterial depletion in anemic mouse
A total of 23 bacterial strains were isolated and characterized based on colony morphology, Gram staining, biochemical tests, and antibiotic susceptibility. All strains formed circular colonies with mostly at elevation and entire margins, predominantly white in color. Gram staining revealed most isolates were Gram-positive cocci, while others were Gram-negative bacilli or coccobacilli. Biochemical analysis showed a common pattern of indole-negative, MR-positive, VP-positive, and citrate-negative results, with most strains being urease-positive, catalase-negative, and coagulase-negative — suggesting likely Enterococcus spp. or related enteric bacteria. Among 14 strains tested, 8 were positive in TSI, indicating fermentative capabilities. MacConkey agar revealed one lactose-fermenting Gram-negative bacillus and two non-lactose fermenters. Antibiotic susceptibility testing showed high levels of Methicillin resistance, with a few strains also resistant to Ampicillin, Chloramphenicol, and Tetracycline, though all strains remained sensitive to Vancomycin. CWS strain of control mouse was sensitive to all antibiotics tested. Collectively, these ndings re ect the presence of diverse, partially resistant enteric bacteria with potential clinical signi cance.



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